The pathology and ecology of Verticillium fungicola (Preuss) Hassebrauk.

Matthews, Colin Trevor

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THE PATHOLOGY AND ECOLOGY OF

VERTICILLIUM FUNGICOLA (PREUSS) HASSEBRAUK

submitted by Colin Trevor Matthews

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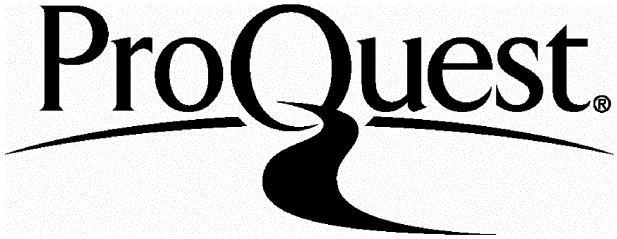

ABSTRACT

The infection process and growth of Verticillium fungicola in mushroom sporophore tissue was studied by light and scanning and transmission electron microscopy. The lesions produced on infection of mature sporophores result from the collapse of host cells brought about by cell wall degrading enzymes produced by the pathogen. The presence of enzymes capable of degrading a mushroom cell wall preparation was demonstrated in vitro.

The mechanisms involved in the development of sporophore deformities were studied using expanding stipe tissue as a convenient model of whole sporophore expansion. Deformities occurred only when rapidly expanding tissue was inoculated and were associated with stimulation of host cell expansion by the pathogen.

V.fungicola showed limited ability to parasitise a number of artificially inoculated Basidiomycete fruit bodies although rust and powdery mildew fungi were readily colonised by the pathogen.

The ecology of V.fungicola was studied to establish likely sources of inoculum as an aid to disease control. Using a specifically developed medium, Verticillium species were isolated from soil, leaf litter, horticultural peat, chalk and insects. Verticillium species were not isolated from a range of wild Basidiomycete fruit bodies and plant pathogenic microfungi.

Major problems were encountered in identifying isolates, due to their morphological variability and to the inadequate and often conflicting taxonomic information available. The majority of the 300 isolates obtained were tested in an in vitro pathogenicity assay but only 3 gave scores equivalent to V.fungicola. Mushroom beds were inoculated with 50 isolates but none reproduced the entire range of symptoms characteristic of the disease.

Benomyl insensitivity in wild populations of Verticillium species was also investigated. In some species (eg. Verticillium psalliotae and Verticillium bulbillosum) the majority of isolates tested were insensitive although sensitive isolates did occur. In others (eg. Verticillium lecanii) all isolates tested were sensitive but selection experiments suggested that insensitive individuals were present in the population.

C O N T E N T S

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

The Pathogen

1. Taxonomy

Preuss(1851) isolated a fungus from an unidentified wild agaric which he named Acrostalagmus fungicola on the basis of the production of hyaline conidia in mucilaginous heads. The genus Acrostalagmus is now generally accepted as synonymous with Verticillium (Issac,1967). Stapf (1889) described a disease of mushrooms which he attributed to Verticillium agaricinum (Link) Corda, the conidial state of Hypomyces ochraceous Pers.

The similarity between the causative agent of 'la môle' disease of mushrooms and a Mycogone species was noted by Constantin and Dufour (1892 a). On sporophores that were distorted but still well defined, two spore types were found: long, cylindrical and occasionally biseptate 'Verticillium' conidia (termed 'Verticillium à grandes spores') and typical Mycogone chlamydospores. Totally deformed sporophores bore smaller 'Verticillium' spores (Verticillium à petites spores). Inoculation of sporophores with either the larger 'Verticillium' spore or chlamydospores resulted in the formation of both these spore types but never the smaller 'Verticillium' type. However, on re-examining the material, Constantin and Dufour (1892 b) concluded, mistakenly, that there was a transition between the two spore types and that the symptoms were due to Mycogone perniciosa Magnus. The production of both distorted and undifferentiated sporophores by a pathogen that conformed to the description of M.perniciosa was confirmed by Viehmeyer (1914).

Malthouse (1901) described a Verticillium species isolated from

diseased mushrooms which he considered identical to the pathogens described by Magnus (1888), Stapf (1889) and Constantin and Dufour (1892 a) all of which had been included in the genus Mycogone. The absence of chlamydospores in Malthouse's isolate was attributed to unsuitable environmental conditions. However, Smith (1924) demonstrated that undifferentiated ('sclerodermoid') sporophores could be produced by two distinct pathogens; M.perniciosa which produced wet, soft sclerodermoid sporophores bearing both verticillate conidiophores and chlamydospores, and a subverticillate species devoid of chlamydospores which produced dry, elastic sporophores. Smith named the latter pathogen Cephalosporium constantinii nov.sp. and considered it identical to the small spored isolate of Constantin and Dufour (1892 a).

This was challenged by Ware (1933) who pointed out that the fungus described by Constantin and Dufour was verticillately branched, whereas C.constantinii was not, and after a detailed study, concluded that the disease symptoms and causal organism corresponded with the description made by Malthouse (1901). Ware named the pathogen Verticillium malthousei nov. sp.

A Verticillium species pathogenic to mushrooms that did not correspond morphologically to V.malhousei, C.constantinii or M.perniciosa was isolated in Denmark by Treschow (1941) and named Verticillium psalliotae nov.sp. This species lacked the erect, verticillately branched conidiophores of V.malhousei and frequently produced a red pigment on artificial culture media. V.psalliotae was first reported in the U.K. by Atkins (1947).

In a study of eleven isolates Fassatiova,(1965) concluded that V.psalliotae and C.constantinii were identical and could be grouped within V.malthousei on the basis of size and shape of the conidia. This view was supported by Issac (1967) who maintained that conidial size and

shape were poor criteria for speciation in Verticillium.

The validity of the current name, Verticillium fungicola (Preuss) Hassebrauk, was established by Gams (1971), who indicated that it predated the name V.malthousei by two years.

2. Dispersal

Unicellular conidia (approximately 3 - 8.5 x 1 - 2.5 µm) are borne on phialides which arise in whorls from erect conidiophores. Conidia are produced successively at the phialide tip and are held together in spherical masses by a water-soluble sticky mucilage (Plate 1). Conidia are primarily splash dispersed. However, the sticky conidia also readily adhere to surfaces and may be dispersed through human agency and by vectors such as mushroom flies and mites (Cross & Jacobs, 1969; Fekete 1967). In wind tunnel experiments, Cross (1971) was unable to demonstrate dispersal of conidia at windspeeds far in excess of those in mushroom houses, but conidia may be aerially dispersed in association with dried, contaminated debris when it is disturbed (Gandy, 1978).

3. Survival

Unlike species of Verticillium parasitic on higher plants, V.fungicola does not produce resting structures, and because the thin walled conidia are poorly adapted to withstand desiccation(Gandy, 1972), relative humidity is probably the most important factor governing survival. Conidia within the spore masses are likely to be protected from drying by the enveloping mucilage and individual conidia that are not deposited on mushroom tissue may survive in moist casing for over 12 months (Cross, 1971). Conidia may also survive under more adverse conditions, for example on dry mushroom house floors(Gandy, 1978), but are likely to do so only when protected from drying by debris. The pathogen can survive

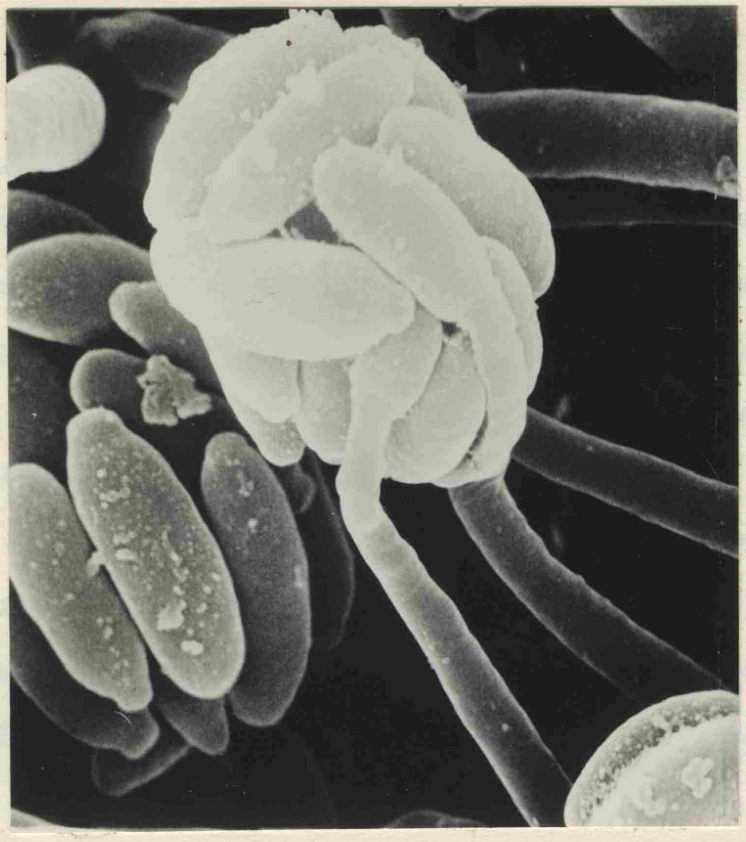

Plate 1

Scanning electron micrograph of a V.fungicola conidial mass.

The mucilage that normally envelops the conidia has been removed during the preparation of the specimen. x 10 000

long periods in dry, infected mushroom tissue (Sinden,1971). The high relative humidity necessary for the cultivation of Agaricus bisporus (Lange) Imbach in mushroom houses favours both the survival and spread of the pathogen.

4. Host Range

Little is known of the ability of V.fungicola to attack fungi other than the cultivated mushroom. All commercial strains of A.bisporus are susceptible to V.fungicola although there is some variation in the degree of susceptibility between strains (Gandy and Spencer,1978; Poppe,1967).

The only published records of V.fungicola from a natural habitat are from an unspecified woodland toadstool (Preuss,1851) and from decaying leaf litter (C.M.I.,1976). In a search for natural hosts of V.fungicola Cross (1971) examined a large number of wild Basidiomycete fungi but failed to isolate the pathogen.

More is known of the host range of the related pathogen V.psalliotae (Treschow) which, as well as parasitising A.bisporus (Treschow,1941) and Agaricus bitorquis (Quel.) Sacc. (Upstone and Carter,1979) has also been reported as a pathogen of the Zygomycete fungus Rhopalomyces elegans Corda (Dayal and Barron,1970) and two graminaceous rusts (C.B.S. culture list, 1963).

The Disease

V.fungicola causes the dry bubble disease of the cultivated mushroom, A.bisporus, and has been reported from most countries where mushrooms are grown commercially. It is economically important and is responsible for annual losses amounting to 2 - 10% of the total value

of the U.K. mushroom crop (Gaze & Fletcher, 1975).

1. Symptoms

The type and severity of symptom produced on infection by V.fungicola is thought to reflect the earliness of infection in relation to the growth and development of the sporophore (Gandy,1972). On fully differentiated sporophores a sunken, brown lesion bearing grey, sporulating mycelium is formed within 24 h under optimal conditions (Plate 2). If infection occurs before full differentiation has taken place, symptoms can vary from slight distortions and swellings to totally undifferentiated masses of tissue several centimetres in diameter (sclerodermoid sporophores). The disease takes its name from these swollen deformities (Plates 3-5). Other characteristic symptoms include peeling of stipe tissues, cracking of cap tissue and locally or totally arrested development of the sporophore cap (Smith, 1924) (Plates 6 & 7). In the absence of secondary bacterial infection, the tissues of infected sporophores remain dry, contrasting with the soggy internal tissue of sporophores attacked by Mycogone perniciosa(Magnus) Delacroix. Most of the surfaces of infected sporophores are covered by sporulating mycelium (Atkins, 1948; Ware, 1933).

2. Interaction of the Pathogen and Host

Conidia of V.fungicola require an exogenous source of nutrients for successful germination and are prevented from germinating in casing by competition for nutrients from bacteria (Cross, 1971). Substances released from growing mushroom mycelium stimulate the germination of conidia and germ tubes then grow towards and contact mushroom hyphae. The germination of conidia on sporophore surfaces is similarly stimulated by exuded nutrients (Vincent-Davies, 1973).

Plate 2

Lesions (arrowed) produced by infection of mature A.bisporus sporophores by V.fungicola.

3

4

5

Plates 3, 4 & 5

Deformities produced on infection of incompletely differentiated A.bisporus sporophores by V.fungicola.

Plate 6 Peeling of stipe tissue (arrowed) and arrested pileus development in sporophores infected by V.fungicola.

Plate 7 Cracking of pileus tissue caused on infection of A.bisporus sporophores by V.fungicola.

Cross (1971) also demonstrated that, after contact has been made, germ tubes grow and the resulting mycelium sporulates in close association with mushroom hyphae, although causing no apparent damage. The growth of V.fungicola along mushroom hyphae and hyphal strands was considered to be a means whereby infection of sporophore initials was assured.

The symptoms of dry bubble are manifested only in the mushroom sporophores and the vegetative mycelium does not appear to be attacked. Growth of the pathogen within the host tissue is intercellular and,in surface lesions, the mushroom hyphae appear deformed and indistinct (Ware, 1933). The observation that the zone of damaged hyphae extended beyond the region of tissue discolouration led Ware to suggest that enzymes were involved in pathogenesis. There is, however, no massive breakdown of tissue such as occurs in M.perniciosa infections where the role of degradative enzymes in pathogenesis has been established (Vincent-Davies, 1973). Nothing is known of the mechanism of the production of deformities and distortions of mushroom tissue by V.fungicola but their expression suggests an interference with the control of cell division and/or expansion in the host.

3. Control

The occurrence of a fungal pathogen on fungal host renders disease control based on selective toxicity difficult. In the past dry bubble control measures consisted of strict farm hygiene combined with dithiocarbamate fungicides such as zineb and mancozeb (Smith, 1970). These were superseded in the early 1970s by the carbendazim fungicide benomyl which gave good and persistent disease control (Gandy, 1972).

However, in vitro insensitivity to benomyl was noticed during early trials (Gandy, 1971; Wuest, 1971) and within three years of commercial use in the U.K., benomyl insensitive strains were widespread

(Fletcher and Yarham, 1976). Some strains that had been isolated prior to the introduction of benomyl also proved to be insensitive (Wuest, Cole and Sanders, 1974) suggesting that large scale and often indiscriminate use of the fungicide rapidly selected these strains, which now predominate. Stringent hygiene, which is necessary to prevent the spread of dry bubble, became lax during the period when benomyl was widely used and consequently, when the fungicide became ineffective, the disease increased in economic importance.

No other fungicide comparable with benomyl has been discovered. The broad spectrum fungicide chlorthalonil is now the most effective commercially available fungicide for dry bubble control (Gandy, 1975) although its careless use can lead to crop damage (Gandy and Spencer, 1976).

During the early stages of an epidemic, physical isolation of diseased sporophores by inverting plastic pots over diseased sporophores (Munns, 1975) or covering them with salt may give some degree of disease control but is unlikely to contain a serious outbreak.

Biological control of dry bubble using Trichoderma viride Fr. has been reported to be as effective and as economic as currently available fungicides in experimental trials (de Trogoff and Ricard, 1976) but has not gained acceptance on a commercial scale. Under certain conditions T.viride can behave as a mild pathogen of mushrooms (Stearne, 1978).

At present, control of the disease can only be obtained through the prevention of outbreaks by the application of stringent hygiene regimes as outlined by Upstone (1975) and Gandy (1978).

Although the disease has been recognised for many years, effective control is still hampered by inadequate knowledge of the basic biology of the pathogen. Little is known about the role of natural sources as

inoculum. Primary outbreaks of the disease often coincide with warm, dry weather and large-scale soil disturbance in the vicinity of the farm (Gandy, 1972) which supports the suggestion that soil is the main reservoir of the pathogen (Kneebone & Merek, 1961). A more hygienic system of growing that takes into account the biology of the pathogen needs to be introduced, since it would be more likely to be effective in disease control. Consequently, the aims of this research are to investigate the natural habitat of V.fungicola and hence to identify possible sources of inoculum and to determine the mechanisms of pathogenesis involved in dry bubble disease.

CHAPTER 2

GENERAL METHODS

Aseptic technique

All operations involving sterile media were performed so as to minimise the likelihood of contamination and were carried out where possible in a laminar flow air cabinet.

Sterilisation of media and extracts

Growth media were generally sterilised by autoclaving for 15 min at 121°C. Where potentially heat labile materials were involved, media or extracts were sterilised by passage through washed Carlson-Ford HP/EKS grade filters followed by filtration through 0.22 µm membrane filters (Millipore Ltd) under aseptic conditions.

Preparation and sterilisation of glassware

All glassware was rinsed with distilled water before use and, when required, was sterilised by dry heat for 3h at 160°C. In studies of the effect of nutrients on conidial germination, glassware was soaked overnight in Decon 90 and thoroughly rinsed in distilled water before use.

Preparation of media

Growth media were prepared in accordance with the manufacturer's or author's instructions. Agar plates were prepared by dispensing approximately 20 ml of the appropriate molten medium into sterile 9 cm plastic Petri dishes.

Agar media

Czapek-Dox agar

Oxoid Czapek-Dox agar (CM97)	45.4 g
Distilled water	1 l

Cell wall polymer media

Described under the appropriate section.

Littman's oxgall agar (Littman,1947)

Peptone (Oxoid bacteriological L37)	10.0 g
Dextrose	10.0 g
Oxgall (Oxoid Ox bile 150)	15.0 g
Crystal violet	0.01 g
Agar (Oxoid No.3 LI3)	15.0 g

Malt agar

Boots' malt extract	20.0 g
Agar (Oxoid No.3)	15.0 g
Distilled water	1 l

Nadakavukaren & Horner's (1959) medium

Absolute ethanol	5.5 ml
Streptomycin sulphate (Glaxo)	0.1 g
Agar (Oxoid No.3)	7.5 g
Distilled water	to 1 l

Nash's medium (Nash & Snyder, 1962)

Peptone (Oxoid mycological L40)	15.0 g
KH_2PO_4	1.0 g
$MgSO_4 \cdot 7H_2O$	0.5 g
Streptomycin sulphate (Glaxo)	0.3 g
PCNB (Hoechst, Quintozene, 50% W.P.)	1.0 g
Agar (Oxoid No.3)	20.0 g
Distilled water	1 l

Nutrient agar

Oxoid nutrient agar (CM3)	28.0 g
Distilled water	1 l

Potato-dextrose agar

Oxoid potato-dextrose agar (CM139)	39.0 g
Distilled water	1 l

Prune agar (CMI, 1968)

Prune extract	5.0 g
Lactose	5.0 g
Difco yeast extract	1.0 g
Agar (Oxoid No.3)	30.0 g
Distilled water	1 l

Sorbose medium (Jordan, 1971)

Sorbose	2.0 g
Streptomycin sulphate (Glaxo)	1.0 g
Agar (Oxoid No.3)	10.0 g
Distilled water	1 l

VP medium (Tsao & Ocana, 1969)

Cornmeal agar (Oxoid CM103)	21.2 g
Pimaricin (Gist-Brocades)	0.01 g
Vancomycin hydrochloride (Eli Lilly)	0.2 g
PCNB (Hoechst, Quintozene 50% W.P.)	0.1 g
Distilled water	1 l

Water agar

Agar (Oxoid No.3)	15.0 g
Distilled water	1 l

Medium 17Z (D.G.Gandy, unpublished)

Glycerol	20.0 ml
KNO_3	1.0 g
Na_2HPO_4	9.8 g
Citric acid	4.7 g
Agar (Oxoid No.3)	15.0 g
Distilled water	to 1 l

Liquid media

Czapek-Dox liquid medium

Czapek-Doz liquid medium (Oxoid CM95)	33.4 g
Distilled water	1 l

Mushroom flour medium

Mushroom flour	15.0 g
Distilled water	1 l

The mushroom flour was prepared by drying sporophores at 80°C for 3 days, grinding them and sifting the flour through a 500 µm sieve.

Salts medium used in the mushroom cell wall polymer medium (Malca et al,1966)

KNO_3	1.0 g
$MgSO_4$	0.5 g
$Fe(NO_3)_2 \cdot 9H_2O$	1.5 mg
$ZnSO_4 \cdot 7H_2O$	0.9 mg
$MnSO_4 \cdot 4H_2O$	0.4 mg
K_2HPO_4	11.6 g
KH_2PO_4	18.1 g
Distilled water	1 l

Dilute water agar used in the preparation of soil suspensions

Agar (Oxoid No.3)	1.0 g
Distilled water	1 l

Preparation of buffered media

In studies of the effect of pH on the germination and growth of V.fungicola double strength media were prepared and sterile buffer solutions were added to cooled (c. 45°C) but molten agar or liquid media to give 50% v/v buffer.

Preparation of colloidal chitin for the polymer medium

Crab shell chitin (Sigma Chemical Corp.) was purified by alternately washing for 24 h with molar HCl and NaOH, the cycle being repeated 6 times. The chitin was then slowly dissolved in concentrated HCl and reprecipitated by rapid dilution with a large volume of distilled water with constant stirring. The colloidal chitin was sedimented using a continuous flow centrifuge rotor and washed with distilled water until the pH was approximately 6.0. A sample of the suspension was dried to constant weight to determine the concentration of chitin and the remainder autoclaved and stored at room temperature until required.

Microscopy

Light microscopy. Low power examinations were made with a Wild M5 binocular microscope and high power examinations with a Wild M20 microscope. Thin sections (1 µm) of diseased and healthy tissue, embedded in epoxy resin as described under transmission electron microscopy, were stained with methylene blue and azur II and mounted in Canada balsam.

Scanning electron microscopy (SEM). Segments of cap lesions, c. 2 mm^2 were fixed in osmium vapour for 3 h and washed twice in 0.1N

sodium cacodylate buffer at pH 8.0. The material was dehydrated in a series of acetone/water mixtures, consisting of two changes of 50, 70, 90, 95 and 100% acetone, 1.5 h in each. The segments were placed overnight in dry acetone, then critically point dried and coated with gold. Specimens were examined in a Jeol CX 100 transmission/scanning electron microscope.

Transmission electron microscopy (TEM). Sections of lesions and healthy tissue, c. 2 x 1 x 0.5 mm, were placed in Karnovsky's fixative (Karnovsky, 1965) for 2 h, washed 5 times at 20 min intervals with 0.1N sodium cacodylate buffer (pH 7.2) and post fixed with osmium for 2 h. The sections were washed twice in buffer for 20 min and progressively dehydrated in an ethanol/water series; 20 min treatment in 2 changes of 20, 50, 70, 80, 90 and 100% ethanol. The sections were progressively infiltrated with Spurr's (Spurr, 1969) resin by 16 h treatment in 1:3 resin/EtOH, 1:1 resin/EtOH, 3:1 resin/EtOH, followed by 2 changes of 100% resin. The tissue blocks were orientated in fresh resin in embedding capsules and the resin was polymerised at 70° for 8 h. Thin sections, 80-100 nm were cut on an LKB ultratome and were examined with a Jeol 100S transmission electron microscope.

Spore germination and growth measurements

1. Linear growth on agar media

Plates of the appropriate medium were prepared and two diameters were drawn at right angles to each other on the bases of the Petri dishes. The plates were inoculated at the intersection of the axes with a 5 mm diameter plug of agar and mycelium, cut from the margin of a 7 d old colony of the test fungus. Three replicate plates were prepared per treatment. The plates were incubated under the appropriate

regime and colony diameters were measured along both axes at regular intervals. Mean colony diameters were calculated from the 6 diameters at each measurement and were corrected by subtracting the inoculum diameter.

2. Germination

Preparation of conidial suspensions

Conidial suspensions were prepared by flooding 7 d old colonies of the appropriate fungus with c. 15 ml of sterile distilled water and disrupting the colony surface with a flamed, bent glass rod. The suspension was passed through sterile Whatman's No.1 filter paper to remove mycelial fragments and the conidial concentration was determined using a haemocytometer.

Because conidia of V.fungicola have a partial nutrient requirement for germination, (Cross, 1971) it was important that in studies of the effect of particular nutrients on germination, the conidia were free from contaminants which may have entered the suspension from the agar media. To achieve this, the conidia obtained from one plate were centrifugally washed twice with a total of c. 5 ml of sterile distilled water.

Germination tests

Germination tests were performed on sterile cavity slides, incubated at 20°C in Petri dishes lined with moistened filter paper to maintain a high humidity. Except where stated, three replicates were set up per treatment.

Controls were of two types; (a) conidia suspended in sterile distilled water to assess stimulatory effects of test chemicals and (b) suspended in liquid Czapek-Dox medium to check conidial viability. The germination of conidia in Czapek-Dox was always between 95 and 100%

after 24 h at 20°c.

The estimate of germination used in these tests was the mean proportion of 200 randomly chosen conidia per replicate that had germinated after 24 h. Germination was judged to have occurred when the germ tube exceeded the length of the conidium.

Germ tube length was determined as the mean length of 50 randomly chosen sporelings per replicate.

For statistical treatment, the results were arc-sine transformed (Anscombe's modification) when the percentage germination in a particular test approached 0 or 100%.

Despite precautions to ensure standard conditions for the germination tests, the results were often variable, particularly in the water controls. This probably reflects an inherent variation in germinability within the conidial population.

Maintenance of cultures

Cultures of V.fungicola maintained on PDA slopes stored at 1°C, were transferred to fresh slopes at approximately 2-monthly intervals. Isolates of V.fungicola were periodically tested for pathogenicity towards cut sporophores but there was no evidence of loss of pathogenicity in any isloate after prolonged subculturing.

Unless otherwise stated, V.fungicola isolate G3 was used in experimental work. This isolate has been in culture at the GCRI for approximately 4 years.

Commercial cultivation of A.bisporus

In commercial practice, the substrate for the growth of mushrooms is composted vegetable matter with organic and chemical amendments. The compost must supply all the requirements for growth of the mushroom mycelium, ie carbon, nitrogen, minerals and vitamins in the correct

proportions under suitable conditions of oxygenation, moisture and pH. Colonisation of untreated organic substrates by mushroom mycelium is poor and the composting process enhances colonisation by modifying the numbers and types of competing micro-organisms (Atkins, 1974).

The main carbon source used in the preparation of mushroom composts in the U.K. is cereal (usually wheat) straw to which various supplements are added to increase the nitrogen content. The straw is most commonly used in the form of horse manure, but shortages of this product have led to the development of 'synthetic' composts, still based largely on wheat straw, but to which materials as diverse as molasses, potato waste, urea and seed meals are added. Gypsum is often added because calcium is important in determining both the pH and the physical structure of the compost (Chang & Hayes, 1978).

In the composting process, wetted manure or straw and supplements are made into a stack which is regularly turned to introduce oxygen and to assist in physical breakdown. A succession of bacteria, fungi and actinomycetes occurs within the compost, thermophilic species being particularly active in degradation. Composting of the stack continues for about a week after which time the compost is still unsuitable as a growth substrate because of high concentrations of ammonia which are toxic to the mushroom.

The compost is filled into wooden trays and its temperature is allowed to rise to 50 - 60°C under controlled conditions of heating and aeration for 4 - 8 days (a process known as 'peak heating' or Pasteurisation). This completes the composting process by enabling thermophilic microorganisms to reduce the ammonia concentration and to increase the protein and soluble carbohydrate content of the compost. This renders the substrate more selective for mushroom growth, whilst the high temperature generated kills many pests.

When the compost has cooled, it is inoculated ('spawned') with mushroom mycelium that has been grown on cereal grains under aseptic conditions. The grain is mixed throughout the compost and mushroom mycelium fully colonises the compost in about 2 weeks at 25°.

To induce fruiting, the compost is covered ('cased') with a 3 cm layer of a mixture of peat and chalk and the temperature is lowered to 18°. The casing is colonised by mycelium and sporophores appear within 2-3 weeks. Fruiting is not continuous but occurs in a series of 'flushes' at approximately weekly intervals.

Ideally at the end of the crop, usually after 6-8 weeks, the trays are heated to 80° ('cooked out') to prevent the carryover of pests and pathogens to succeeding crops. This procedure is, however, not universal and many pest and disease problems can be attributed to its omission.

Sporophores used in experimental work were freshly picked from the GCRI mushroom unit.

The growth stages of A.bisporus sporophores referred to in the text are those described by Hammond and Nichols (1975) (Figure 1).

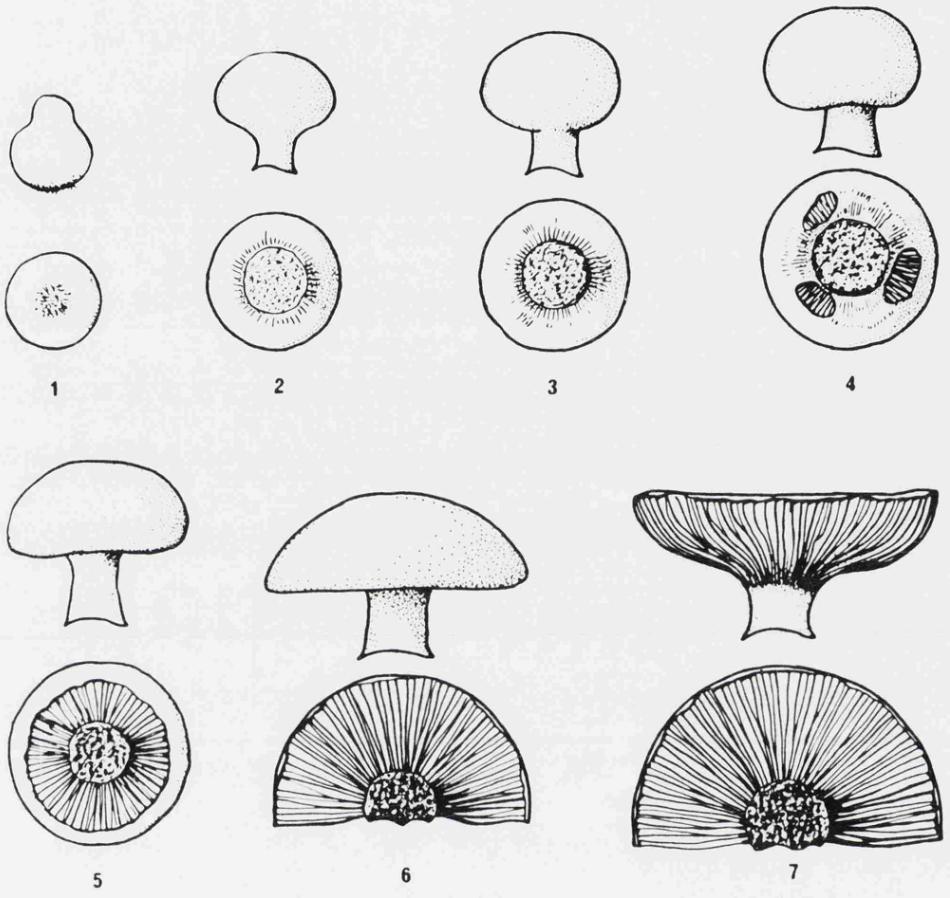

Fig.1. The stages of development of the fruit body of *Agaricus bisporus* (as described by Hammond and Nichols, 1975).

CHAPTER 3

THE EFFECTS OF PHYSICAL FACTORS ON VERTICILLIUM FUNGICOLA

The response of V.fungicola to gradients of temperature, relative humidity and pH were measured to establish limits for the survival of the pathogen in the natural environment. The role of wind in the dispersal of conidia was examined as a supplement to the work of Cross (1971).

TEMPERATURE

Treschow (1941) determined the cardinal temperatures for dry matter production of isolates of both V.fungicola and V.psalliotae. Neither species grew below 8°C and the optima and maxima were 22 & 23° and 30 & 35° for V.fungicola and V.psalliotae respectively. Fekete (1967) obtained similar optima and maxima for linear growth but also recorded some growth at 0°C.

Maximum conidial germination (85-95%) was reported by Wuest and Forer(1971) to occur within 12 hours at 12 and 18° and within 9 hours at 24° on water agar whilst Gandy (1978) obtained 70-95% germination within 16 hours at 20° in distilled water.

Materials and Methods

1. Dry Matter Production

V.fungicola was grown in 250 ml flasks containing 50 ml of Czapek-Dox liquid medium. The flasks were inoculated with a conidial suspension which gave a final concentration of 3.0×10^4 conidia ml^{-1}. Three flasks were placed at 0, 2, 10, 12, 16, 20, 23 and 32° and the

cultures were harvested by filtration onto dried and tared filter paper after 14 days. Dry weights of the mycelia were measured after drying at 80° for 24 hours.

2. Linear Growth

Linear growth of V.fungicola isolate G3 on medium 17Z was determined as described in the General Methods. After 24 h incubation at room temperature, 3 replicate plates were placed at 0, 1, 5, 8, 10, 12, 15 and 24° and colony diameters were measured after 14d.

The linear growth of V.fungicola isolates G3, C1 and S1 was similarly measured but after 18d incubation at 20, 22, 24, 27, 30, 32 and 36° and that of isolates G3, G4, G5, C1 and S1 after 7d incubation at 0, 1, 5, 7 and 10°. In the latter experiment growth was scored as either present or absent. For comparative purposes, V.psalliotae isolate C9 was included in the latter two experiments, the growth medium for which was PDA.

3. Percentage Germination

The percentage germination of nutrient supplemented conidia was measured on cavity slides as described in the General Methods. Three slides were incubated at 0, 1, 5, 10, 12, 15, 20 and 25° and the percentage germination was measured after 24h.

The percentage germination of washed conidia incubated at 0, 1, 5, 8, 10, 12, 15, 18, 22 and 24° was also measured in the absence of exogenous nutrients. Three slides were prepared at each temperature and the controls consisted of conidia supplemented with exogenous nutrients, one slide being placed at each temperature.

Results

The optimum temperature for all three measures of growth of V.fungicola lay between 20 and 24° (Figs. 2-5). There was no linear growth above 30° or below 5° (Table 1) but both conidial germination and dry matter accretion occurred below 1°. Sporulation of all isolates was observed at 5° and above. Conidia did not survive freezing at -20° for 5 days. Linear growth, dry weight increase and germination in the absence of exogenous nutrients reached a plateau slightly below the optimum and a sharp decline above it. V.psalliotae reacted similarly except that it tolerated higher temperatures, some growth occurring at 36°.

The presence or absence of nutrients had little effect on the optimum temperature for conidial germination although the maximum percentage germination was much reduced in the absence of nutrients (Fig. 6). Germination was poor below 10° both in the presence and absence of nutrients.

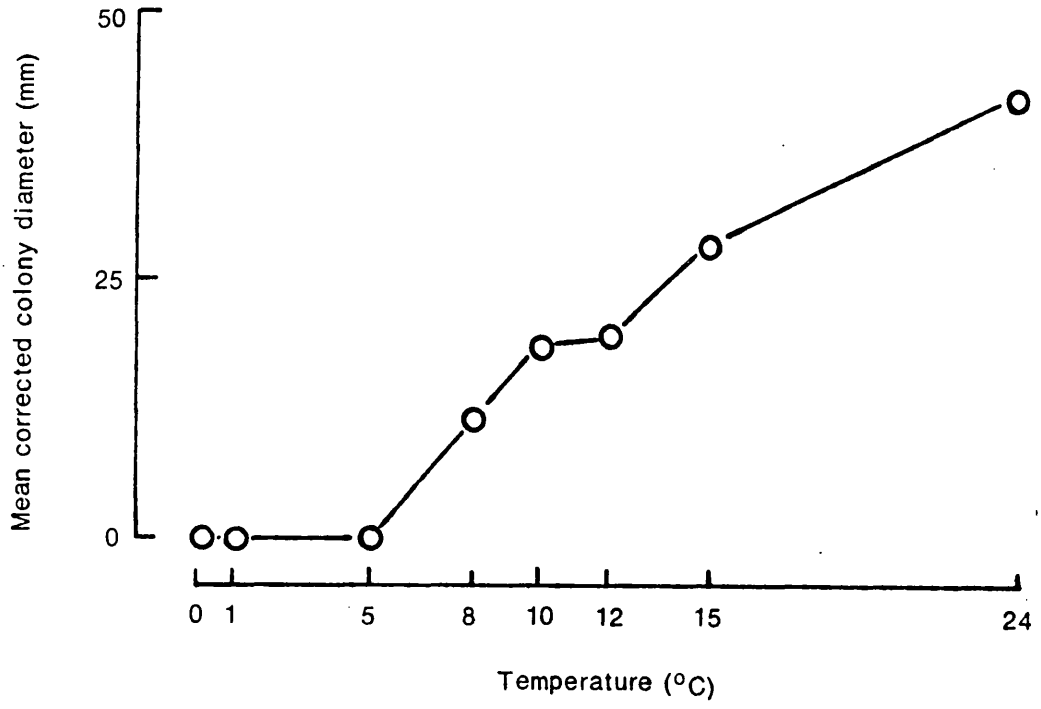

Fig.2. Mean mycelial growth (mm) of *Vf.* isolate G3 on medium 17Z after 14 days at different temperatures.

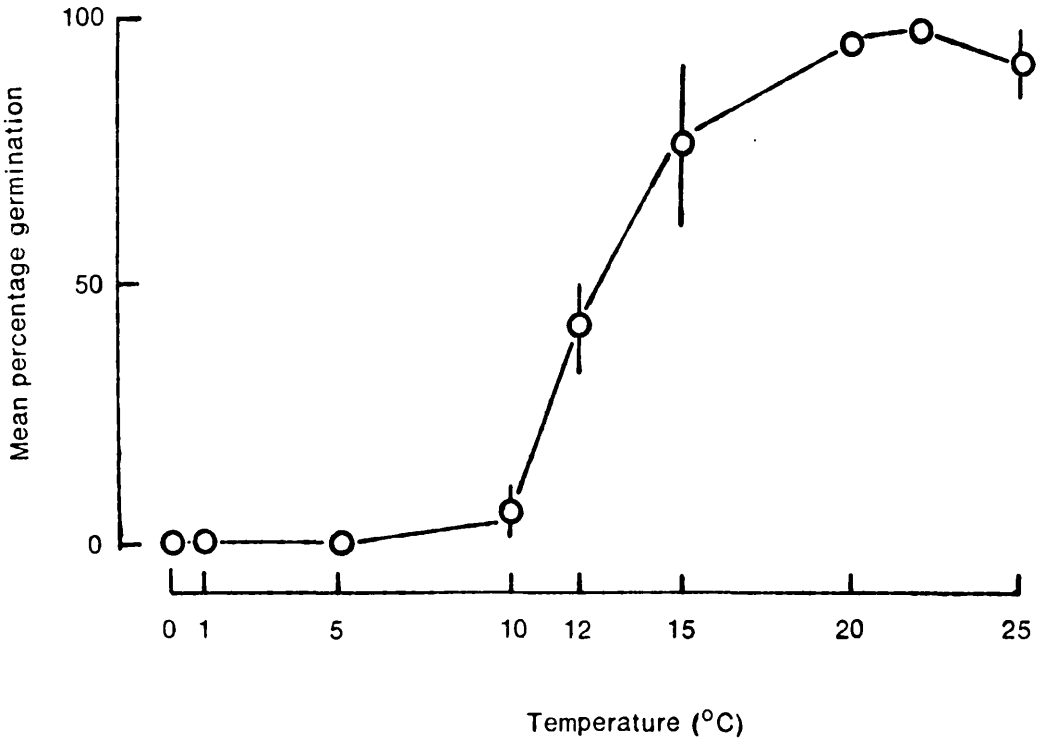

Fig.3. Mean percentage germination of conidia of *Vf.* isolate G3 in liquid Czapek-Dox medium after 24 hours at different temperatures. Bars represent standard error of the mean of 3 replicates.

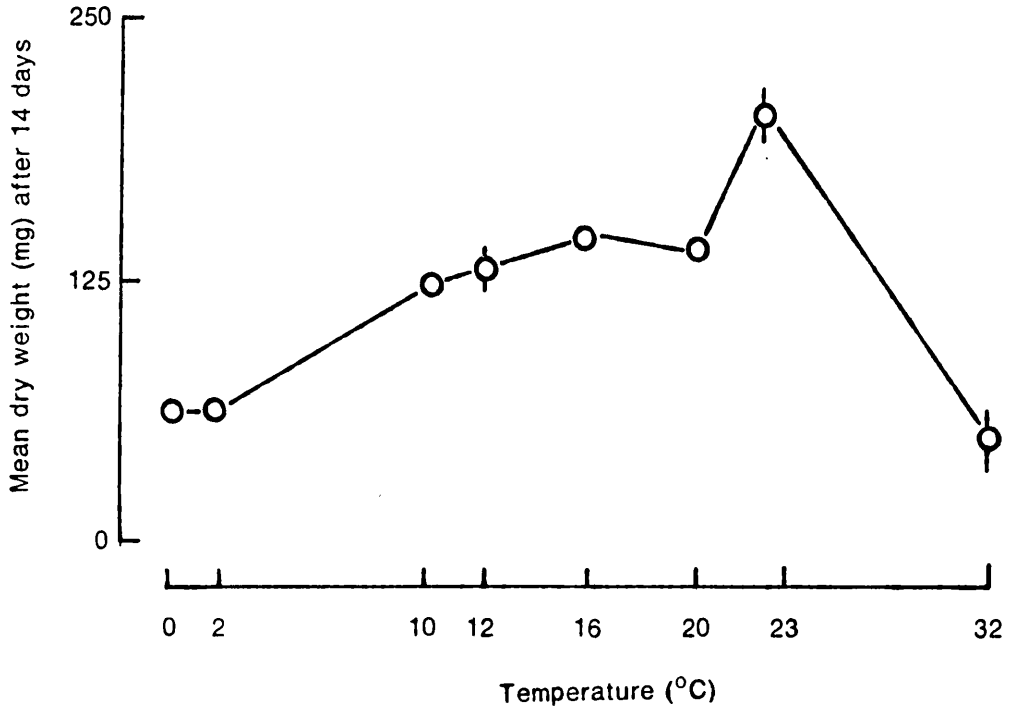

Fig.4. Mean dry weight (mg) of *Vf.* isolate G3 mycelium grown for 14 days in liquid Czapek-Dox medium at different temperatures. Bars represent standard error of the mean of 3 replicates.

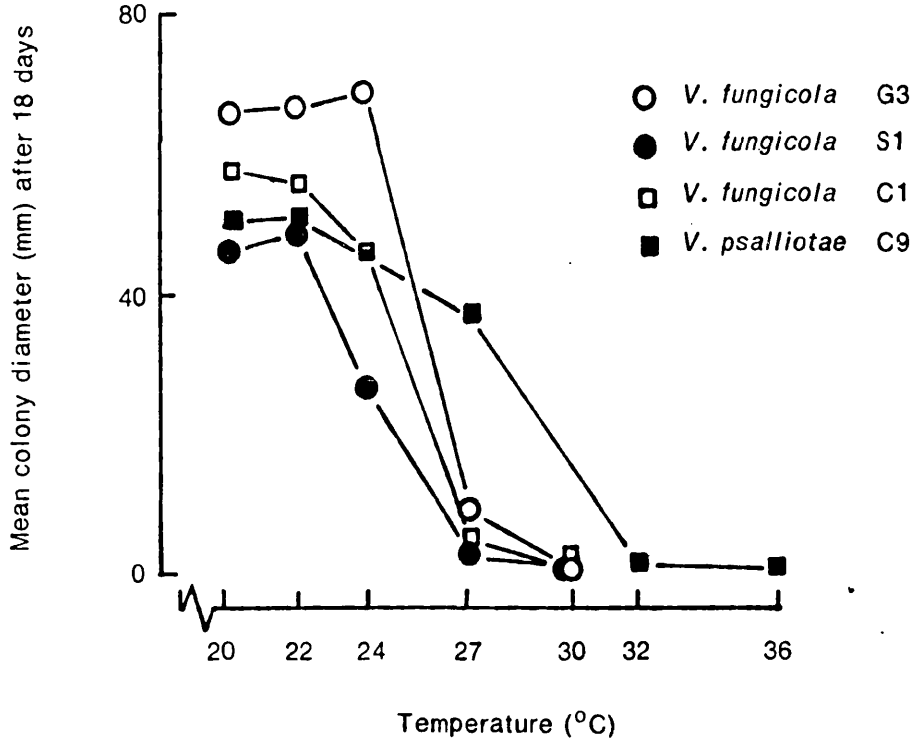

Fig.5. Mean corrected linear growth (mm) of *Vf.* isolates G3, C1 and S1 and *V. psalliotae* isolate C9 on PDA after 18 days incubation at 20 - 34°C.

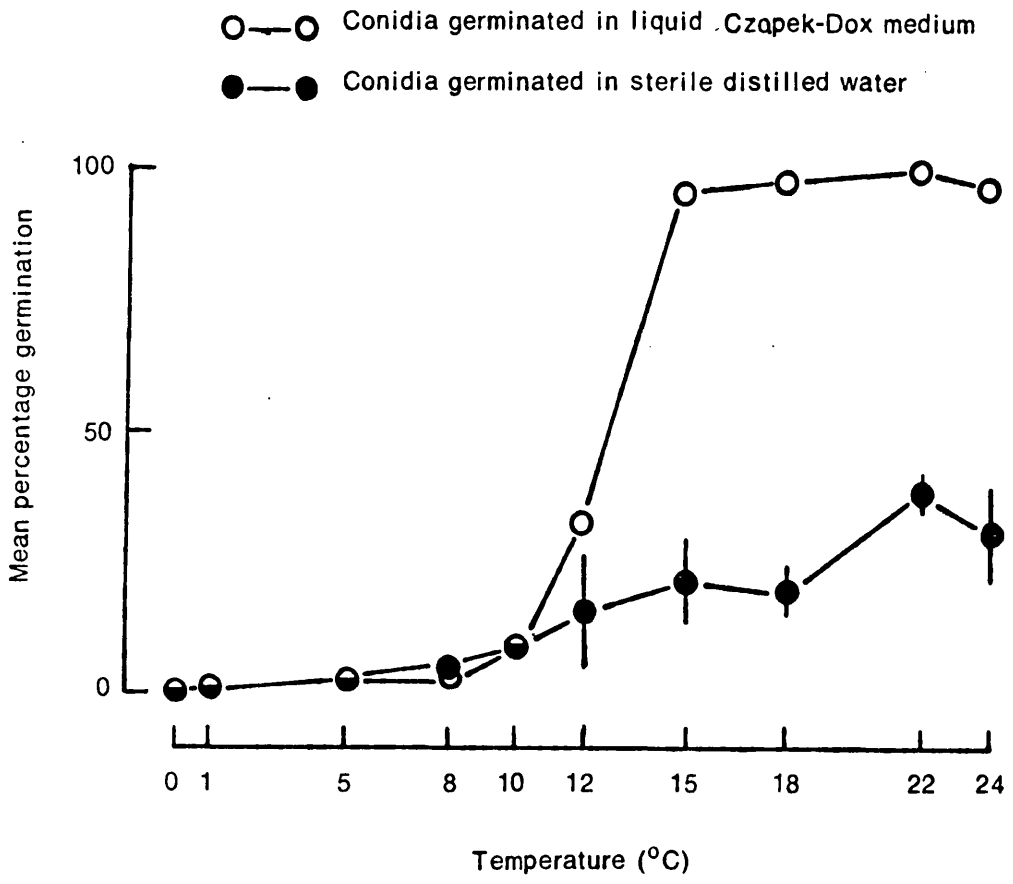

Fig.6. Mean percentage germination of washed conidia of *Vf.* isolate G3 in distilled water and liquid Czapek-Dox controls. Bars represent standard error of the mean of 3 replicates.

Table 1. Growth of Verticillium isolates on PDA after 7 days' incubation at low temperatures.

Isolate	Temperature °C.			
	1	5	7	10
V.fungicola G3	-	+	+	++
" G4	-	+	+	++
" G5	-	+	+	++
" C1	-	+	+	++
" S1	-	+	+	++
V.psalliotae C1	-	+	+	++

\- No growth

\+ Thin mycelium showing some growth away from the inoculum disc.

++ Dense mycelium with good growth away from the inoculum disc.

All isolates sporulated at 5° and above.

Discussion

The optimum and maximum temperatures for growth of V.fungicola and V.psalliotae measured either as dry weight or colony diameter were in agreement with the results of Treschow (1941). There was, however, a relatively large increase in dry weight of V.fungicola at 0 and 2° which Treschow reported were below the minimum temperature for growth. A small proportion of conidia had germinated at 0° after 24 hours and the continuing germination of conidia and mycelial growth over 14 days probably accounted for this increase.

There were differences in the optimum temperature and extent of linear growth of the three V.fungicola isolates, although none grew at 30°. A similar variability in linear growth between isolates in relation to temperature was found by Lambert (1973) who also demonstrated that, whilst isolates of V.fungicola from regions with a high mean summer temperature grew well at 30°, there was characteristically little growth of isolates from northern Europe (England and Switzerland) at this temperature.

The response of conidial germination to temperature was similar to that of linear growth, although germination was less sensitive to temperatures close to zero.

The occurrence of a plateau between 10 and 20°C in the dry weight, germination and linear growth curves suggests that this response is due to a fundamental effect on the physiology of V.fungicola rather than an artefact. However, the reason for the plateau is not clear.

RELATIVE HUMIDITY

The conidia of V.fungicola are thin walled and appear poorly adapted to withstand desiccation. Relative humidity might therefore play an important role in the survival of conidia in the natural environment.

Materials and Methods

Relative humidity chambers were constructed from 1 lb honey jars (Plate 8). Two GEPE photographic transparency holders were fixed into the lids by means of 1 cm lengths of looseleaf binding strip which were glued to the lid lining. Squares (4x4 cm) of boiled dialysis membrane, previously autoclaved in Czapek-Dox liquid medium to act as a nutrient source were clipped into the transparency holders and dried in a laminar flow cabinet either before or after inoculation. For the former, conidia were transferred from an agar colony using a nichrome loop, and for the latter, a conidial suspension was applied with a sable brush. The necks of the jars and lid margins were smeared with petroleum jelly to provide an airtight seal. Relative humidities of 65, 70, 75, 80, 85, 90, 95, 97, 98, 99 and 100% were maintained in the chambers using 100 ml of the appropriate glycerol-distilled water mixture (Carson,1931). In some experiments, 92% r.h. was required and this was maintained with a saturated solution of potassium nitrate. After assembly, the jars were placed in a shaking incubator at 25° and slowly rotated to prevent stratification of r.h. The r.h. and temperature within each jar was measured using a small humidity and temperature sensor (Novasina, type 83E) inserted into the jars through a modified lid. The temperature recorded was 25 ± 0.1° and the r.h. was usually within ±1% of the calculated value. Equilibrium r.h. was re-established within an hour of replacing the lid.

Plate 8

The relative humidity chamber.

For details of the construction, see the text.

1. Relative Humidity and Germination of Conidia

Three series of experiments were done using the r.h. chambers:

a) to determine the percentage germination in 24 h at different humidities using both application methods;

b) to determine the time taken for germination at different humidities using conidia applied to the membranes in suspension;

c) to determine the effect of exposure to different humidities for different times, followed by 24 h at 100% r.h. Conidial suspensions were applied to membranes and exposed to 65, 75, 85, 95 and 100% r.h. for 2, 8, 16 and 24 h prior to final incubation at 100% r.h. Germination was estimated from counts of 200 conidia on each of the 2 membranes. To compare the results of the different experiments, the percentage germination was expressed as a percentage of the 100% r.h. controls.

The effects of low (≃1%) r.h. on conidial germination were studied by applying a conidial suspension to nutrient supplemented dialysis membranes which were then dried in a laminar flow cabinet. The membranes were exposed to silica gel in a desiccator at room temperature for 20, 44, 52 and 60 h, then placed on Czapek-Dox agar and incubated at 20°. After 24 h the percentage germination and germ tube length of 60 randomly chosen sporelings were measured. Control conidia were not exposed to silica gel but were incubated on Czapek-Dox agar throughout. The percentage germination results were subjected to analysis of variance.

For longer (up to 108 h) exposures of conidia to silica gel, visible growth of mycelium on the membranes was scored as either present or absent after 24 h incubation on Czapek-Dox agar.

2. Relative Humidity and Germ Tube Growth

A conidial suspension was applied to nutrient supplemented dialysis membranes which were then placed on Czapek-Dox agar and incubated for 24 h at 20°. The percentage germination and germ tube lengths of 100 randomly sampled sporelings were measured and the membranes were then exposed either to silica gel for 8 h or to 65, 85 and 100% r.h. for 24 h before being returned to Czapek-Dox agar for a further 24 h. Percentage germination and germ tube lengths were assessed again and were compared with the measurements made before exposure using Student's test. Germinated conidia were also exposed to silica gel for periods of up to 124 h before being returned to Czapek-Dox agar and scored for mycelial growth 24 h later. Control conidia, not exposed to low r.h., were incubated throughout on Czapek-Dox agar.

Results

1. Conidial Germination

In the r.h. chamber experiments, the germination of conidia exposed to 100% r.h. for 24 h varied from 67-92%.

Figures 7 and 8 show the effect of 24 h exposure to a range of r.h. and compare the two methods of application of conidia to the membranes. Although germination decreased with decreasing r.h. in both instances, the effect was more marked at 95-100% r.h. with conidia applied directly to the membrane. The experiment was repeated and gave similar results. Conidial germination only occurred above 95% r.h., but increased progressively with increasing r.h. (Figs. 9 & 10). After 144 h incubation at 85% r.h. and below, germination did not exceed 1%.

When conidia were exposed to a range of r.h. for different times and then incubated at 100% r.h. for 24 h, the proportion of viable

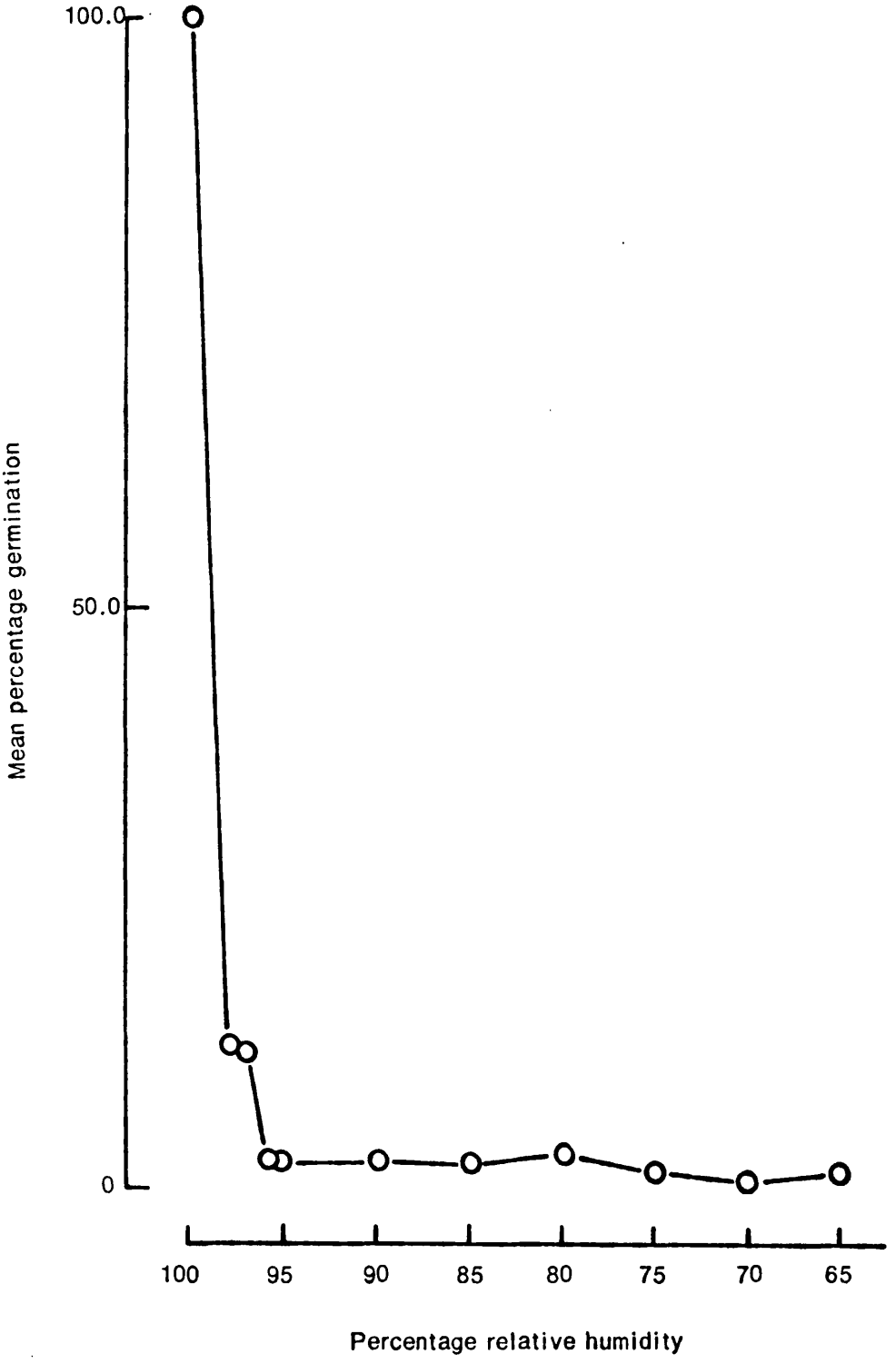

Fig.7. Mean percentage germination of *Vf.* isolate G3 conidia after 24 hours exposure to a range of rh. at 25°C. Conidia were applied to dialysis membranes directly from the colony surface using a nichrome loop. Bars represent standard error of mean of 2 replicates.

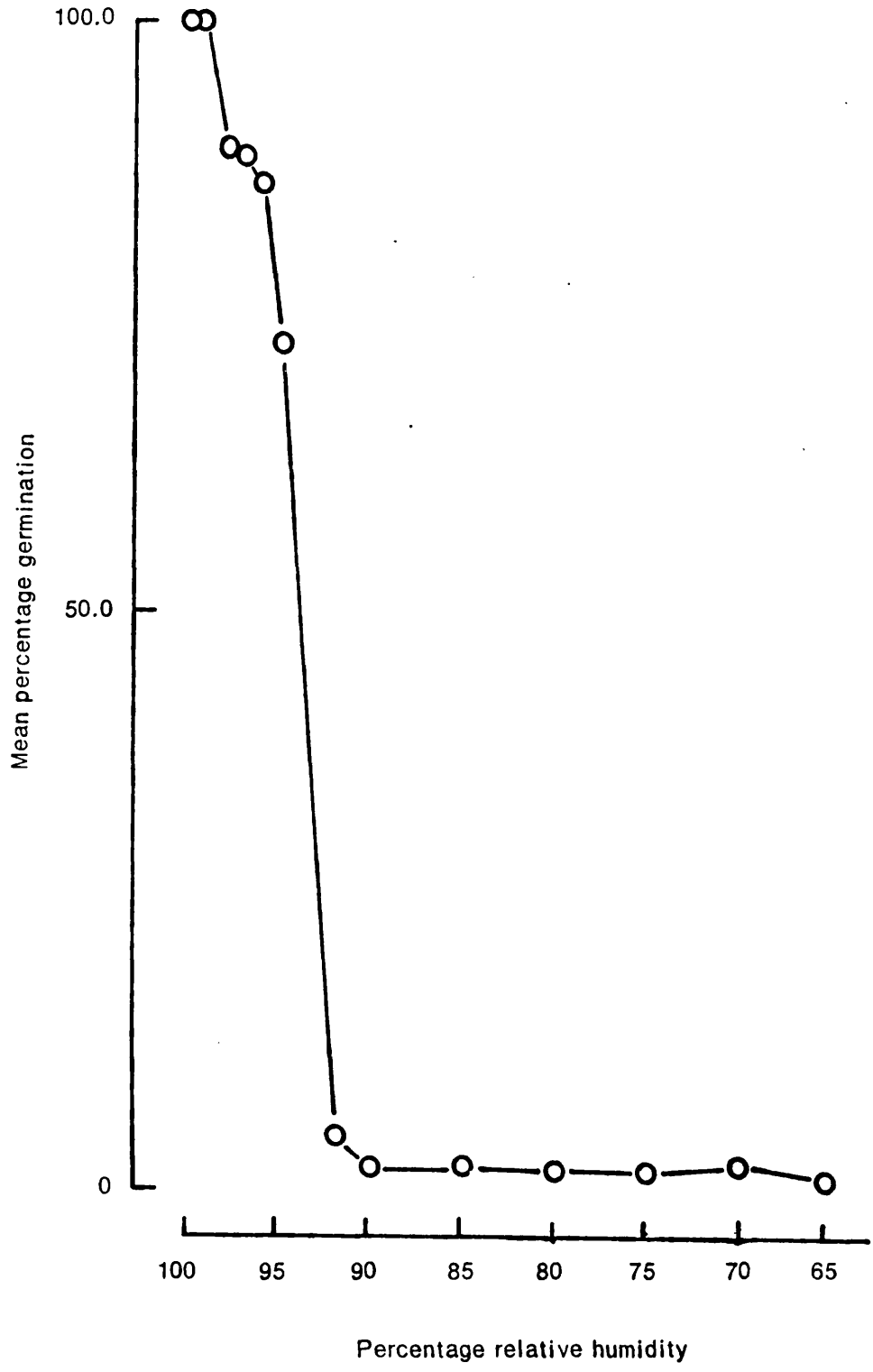

Fig.8. Mean percentage germination of *Vf.* isolate G3 conidia after 24 hours exposure to a range of rh. at 25°C. Conidia were applied to dialysis membranes in suspension using a camel hair brush. Bars represent standard error of mean of 2 replicates.

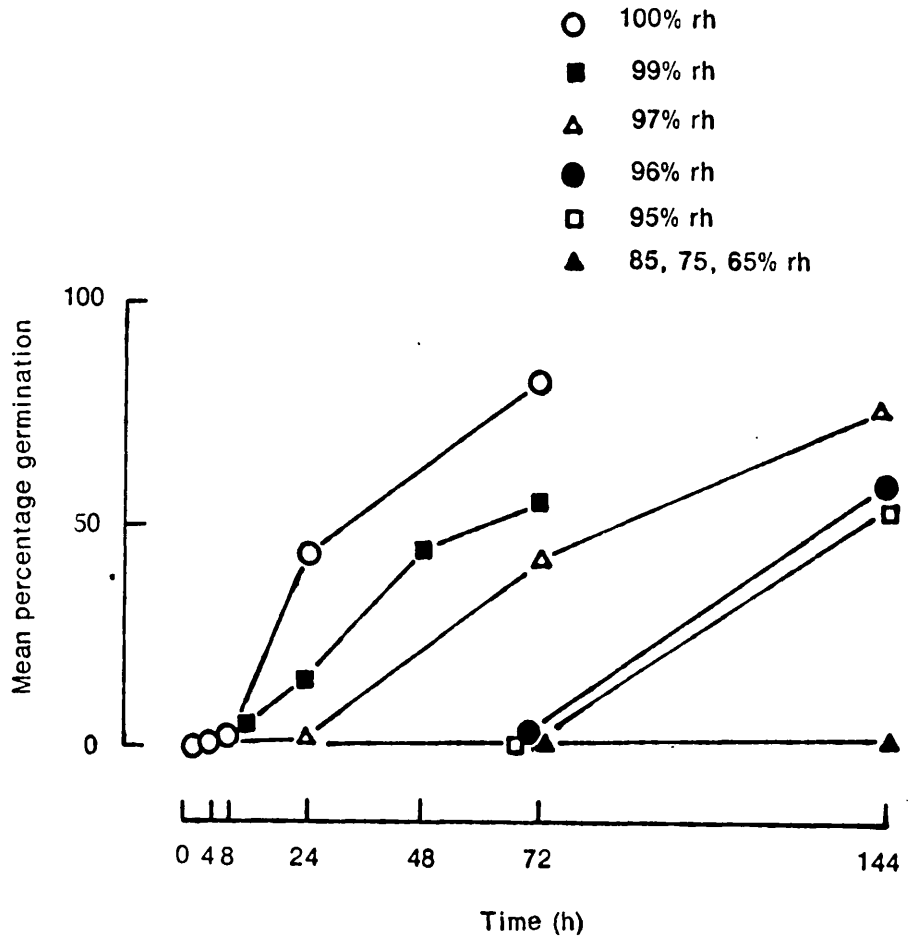

Fig.9. The relationship between relative humidity and the time taken for germination of *Vf.* conidia at 25°C.

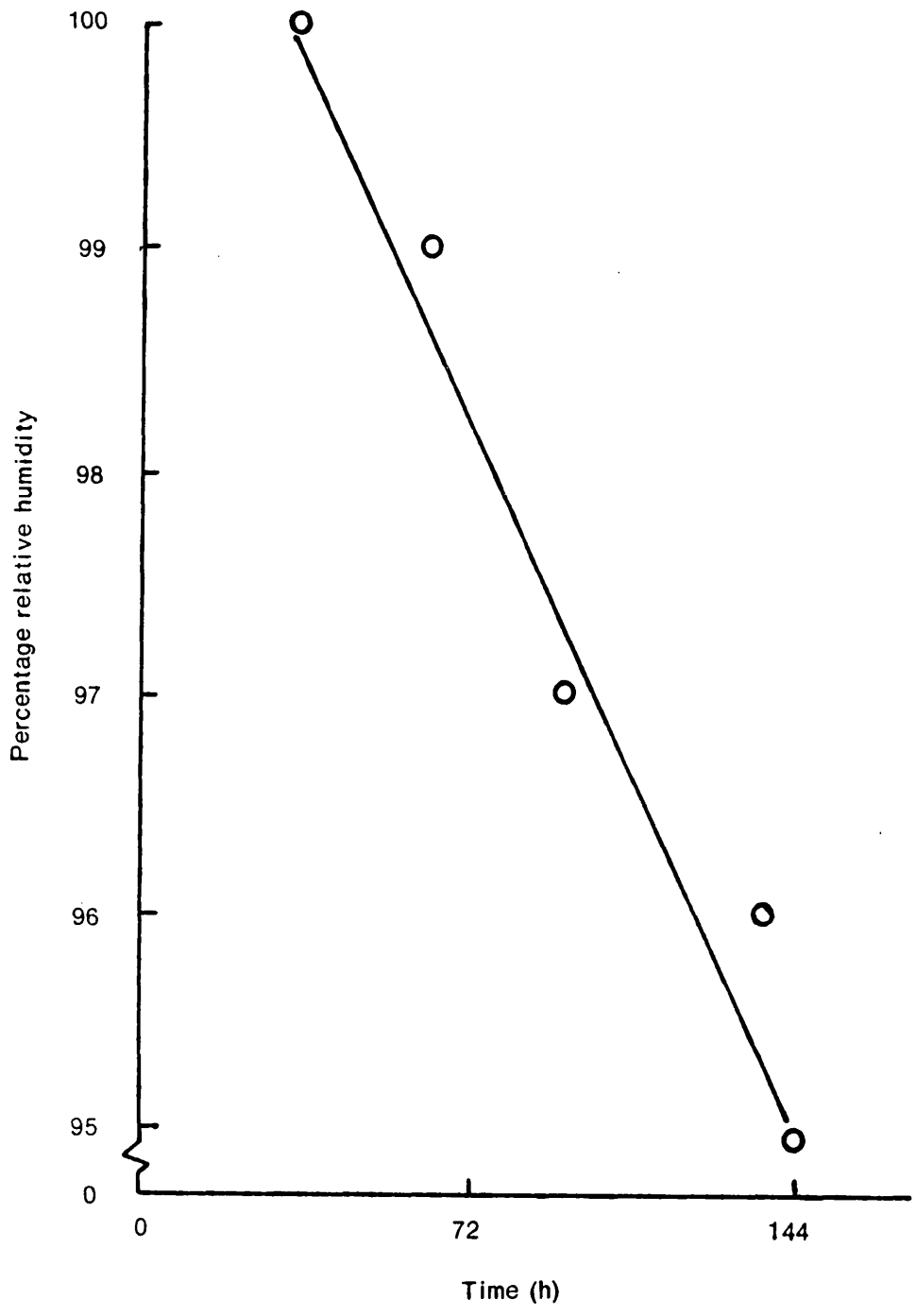

Fig.10. The relationship between rh. above 95% and the time required for 50% germination of *Vf.* isolate G3 conidia at 25°C.

conidia decreased with decreasing r.h. and increasing time of exposure (Fig. 11).

Both the percentage germination and germ tube growth decreased with an increasing exposure to silica gel (Table 2).

Table 2. The effect of exposure to silica gel on germination of conidia and germ tube length

Exposure time (h)	Mean % germination SE	Mean germ tube length (μm) SE
0	98.5 ± 1.3[a]	ND
20	86.5 ± 1.3[a]	110.0 ± 7.2
44	32.6 ± 2.4[a]	48.2 ± 7.1
52	13.0 ± 1.2[a]	48.2 ± 19.0
60	11.9 ± 0.7	58.2 ± 3.2

a Significantly different at $P = 0.05$

ND Not determined as germ tubes were too long to be measured individually.

Conidia exposed to silica gel for periods of up to 108 h produced visible growth on the membranes after incubation on Czapek-Dox agar for 24 h.

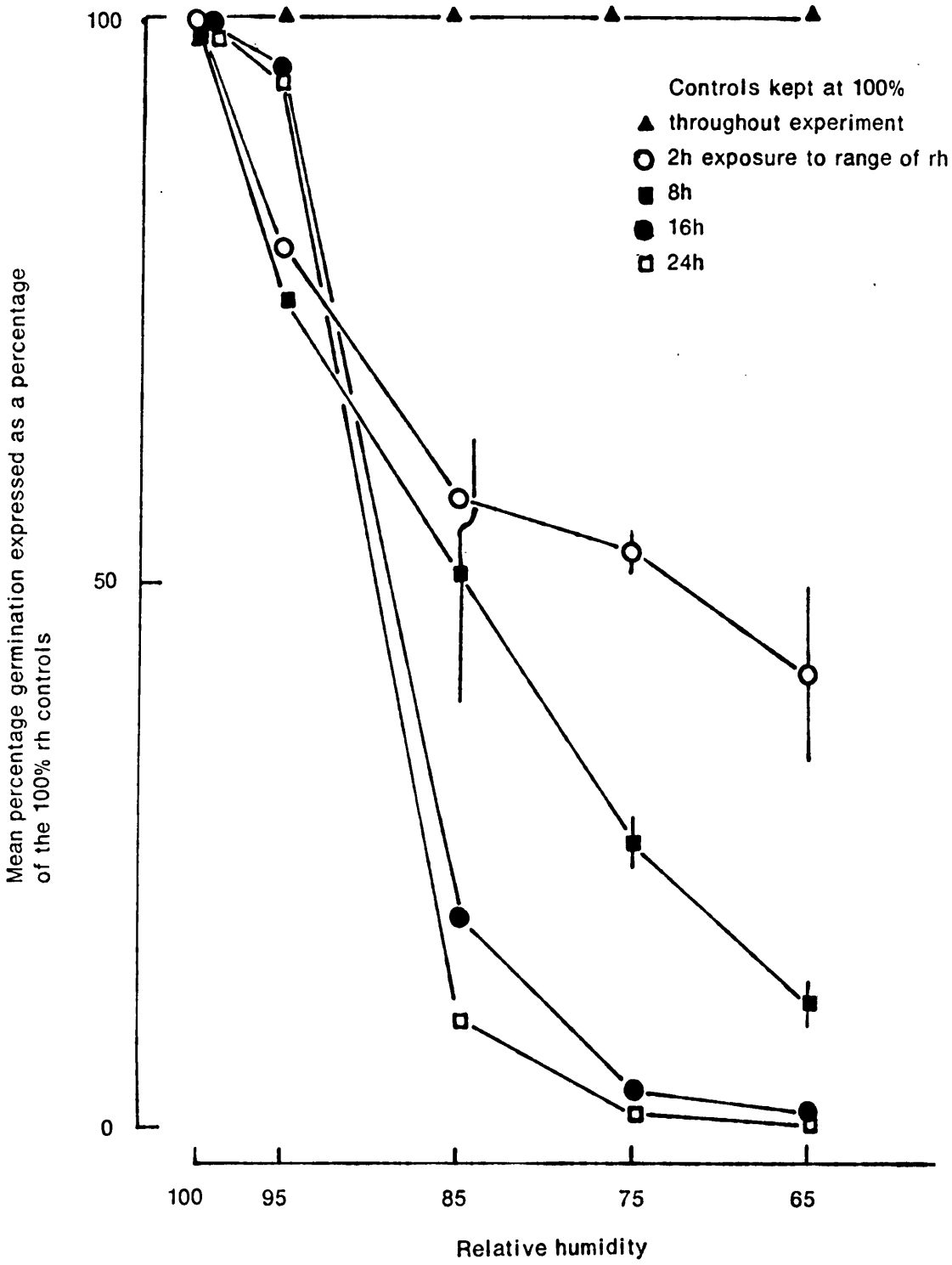

Fig.11. Viability of *Vf.* isolate G3 conidia after exposure to 5 rhs for 2 – 24 hours followed by incubation at 100% rh for 24 hours at 25°C. The results are expressed as a percentage of the 100% rh controls. Bars represent standard error of mean of 2 replicates.

2. Germ Tube Growth

The germ tubes of 24 h old sporelings continued to grow on being returned to Czapek-Dox agar after 8 h exposure to silica gel (Table 3).

Table 3. Germ tube growth after exposure to silica gel for 8 hours

Treatment	% germination SE	Mean germ tube length (μm) SE
24 h on Czapek-Dox agar.	95.6 ± 1.5	[a] 12.9 ± 0.6
24 h on Czapek-Dox agar 8 h exposure to silica gel and 24 h further incubation on Czapek-Dox agar.	96.2 ± 1.6	[a] 64.8 ± 5.7
CONTROL 56 h on Czapek-Dox agar.	95.0	Too long to measure.

a Significantly different at $P = 0.05$

Exposure of germinated conidia to silica gel for up to 124 h did not prevent the subsequent growth of germ tubes on being returned to Czapek-Dox agar.

Germinated conidia exposed to gentle desiccation over glycerol/water mixtures were however more sensitive to r.h. (Table 4).

Table 4. Germ tube growth after exposure to glycerol/water mixtures for 24 h.

	% r.h.		
	100	85	65
Mean % germination after 24 h	99.0	99.0	99.0
Mean germ tube length after 24 h. (µm) ± SE	113.3 ± 6.0	110.4 ± 7.7	*111.0 ± 6.0
Mean % germination after exposure and 24 h further incubation on Czapek-Dox agar	ND	99.0	99.0
Mean germ tube length after exposure and 24 h further incubation on Czapek-Dox agar. (µm) ± SE	ND	800	*124.2 ± 7.8

* Not significantly different at $P = 0.05$.

ND Not determined as the germ tubes were too long to measure and obscured the individual conidia.

After 3 days' further incubation on Czapek-Dox agar there was little growth on the membrane exposed to 65% r.h. although the membranes exposed to 85 and 100% r.h. were covered with a dense mat of mycelium.

Discussion

The results of the r.h. chamber experiments confirm the report by Gandy (1972) that individual conidia of *V.fungicola* are highly susceptible to low r.h. Although a few conidia germinated at all humidities

tested, a large proportion germinated only at r.h. above 90% (spore suspension) and 95% (direct inoculation).

The cause of this discrepancy could be attributed to the time taken for the liquid in which the conidia were suspended to evaporate. Thus the actual time of exposure to the test r.h. would vary, depending on r.h. but would be less than 24 h except for spores at 100% r.h. Another factor may be the initiation of germination during the preparation of the conidial suspension. The first morphological change associated with germination in V.fungicola is the swelling of conidia and measurements of the length/breadth ratio of germinating conidia show that this occurs within a few hours of suspension (Fig. 12). This indicates that the biochemical changes associated with initiation of germination are extremely rapid and could well occur during the time taken for the preparation of a conidial suspension and its application to the membrane.

The results of these experiments are in agreement with the data presented by Yarwood (1978) for several other Hyphomycetes which also require either high r.h. or free water for germination.

Low r.h. also delayed germination. With 144 h exposure, little germination occurred below 85% r.h. whilst above 95% r.h. the time taken to reach 50% germination decreased linearly with increasing r.h. Snow (1949) reported that the latent period for the germination of the majority of conidia of Aspergillus repens increased from 2 - 3 days at r.h. of 88% and above to 30 days at an r.h. of 76%, so that it is possible that further germination would have occurred had the experiment continued beyond 6 days.

The sensitivity of V.fungicola conidia to low r.h. is underlined by the fact that a few hours exposure to r.h. below the threshold for germination caused a large reduction in viability. In view of this, the relative insensitivity of conidia to exposure to extremely low r.h.

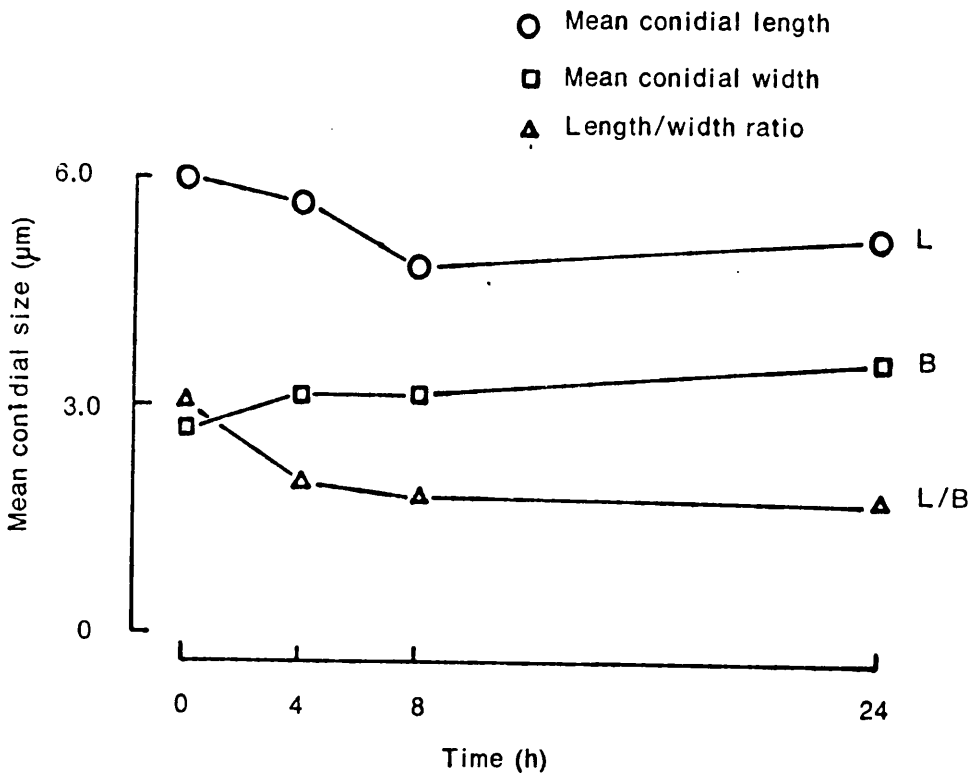

Fig.12. Variation in the length, breadth and length/breadth ratio of 50 randomly chosen conidia of *Vf.* isolate G3 germinating in 0.1% water agar.

determined by silica gel was surprising.

It is again possible to explain this discrepancy in terms of differences in the speed of drying of the conidia in the two methods. In the r.h. chambers, the conidia were subjected to fairly gradual drying even at the lowest r.h. used (65%) whereas over silica gel, desiccation was very rapid. Conidia exposed to 95% r.h. and above are able to initiate and complete the germination process by absorbing moisture from the atmosphere (Snow, 1949), the length of time taken for germination depending on r.h. At r.h. below 95%, conidia may be able to absorb sufficient moisture to initiate the irreversible process of germination (Sussman, 1966) but be unable to germinate because of a lack of water required for the extrusion of the germ tube. In this non-dormant state, the damage caused to the biochemical pathways by desiccation of the conidium may be irreparable and consequently viability is lost. Conidia that are dried rapidly are likely to be unable to initiate germination, are protected from damage, and are thus able to germinate on return to a favourable environment. Conidia of Monilinia fructicola have similarly been shown to be less susceptible to rapid as opposed to gentle drying (Good and Zathureczky, 1967).

The desiccation of germ tubes of V.fungicola over silica gel had little effect on subsequent growth under conditions of rapid rehydration and abundant nutrients. However, as was found with ungerminated conidia, gentle drying such as is likely to occur in the natural environment had a more harmful effect on the survival of germ tubes. Similar results were obtained with germ tubes of Botrytis cinerea, Colletotrichum musae and Monilinia fructicola by Good and Zathureczky (1967).

In the natural environment, drying of conidia is likely to be gentle and the majority are unlikely to survive for long periods in the absence of protection from substrates containing water.

pH.

The effect of pH on dry matter production by both V.fungicola and V.psalliotae in unbuffered media was examined by Treshow (1941). Both fungi grew over a wide range of pH (2.6 - 7.4) but whereas V.fungicola showed a distinct optimum at pH 5.6 and caused little change in the pH of the growth medium, V.psalliotae produced a large amount of acid and a true optimum could not be determined.

Materials and Methods

In all experiments, culture media or nutrient solutions were buffered with 50% v/v of the appropriate buffer. Methods for assessing linear growth or percentage germination on cavity slides are described in the General Methods section.

1. Linear growth

Colony diameters were measured after 14 days' growth on Czapek-Dox agar buffered either with citrate-phosphate buffer at pH 2.8, 3.8, 4.8, 5.8, 6.8 and 7.8, glycine-HCl buffer at pH 2.2 and 3.2 or phosphate-phosphate buffer at pH 5.0, 6.0, 7.0 and 8.0. The pH of unbuffered controls was approximately 6.0 and there was little change in the pH of the medium during growth.

2. Percentage germination and germ tube growth

The percentage germination of washed conidia incubated in liquid Czapek-Dox medium buffered either with citrate-phosphate buffer at pH 2.8, 3.8, 4.8, 5.8, 6.8 and 7.8 or with phosphate-phosphate buffer at pH 5.0, 6.0, 7.0 and 8.0 was measured after 24 h at 20°.

The effect of pH on the percentage germination in the absence of

exogenous nutrients was also determined using citrate-phosphate buffer. The pH of unbuffered controls was again 6.0 and there was no change in the pH of the suspension during germination. The percentage germination results were arc-sine transformed and subjected to an analysis of variance.

Results

1. Linear growth

V.fungicola grew over the entire range of pH examined. On citrate-phosphate buffered medium there was a slight optimum in linear growth at pH 4.8 and growth at all pHs was greater than in the unbuffered controls (Fig. 13a). On the glycine-HCl and phosphate-phosphate buffered media, growth was poor at pH 2.2 but was little affected by pH from pH 3.2 to 8.0, being similar to that on the unbuffered controls (Fig. 13b).

2. Percentage germination and germ tube growth

When conidia were germinated in liquid Czapek-Dox medium buffered with citrate phosphate buffer, the pH of the medium had little effect on the percentage germination (Fig. 14a). With phosphate-phosphate buffer, the percentage germination was greatest at pH 5.0 but the differences in germination between pH 6.0 and 8.0 were not significant ($P = 0.05$) (Fig. 14b). In both cases the percentage germination was greatest in the unbuffered controls.

In the absence of exogenous nutrients, there was a clear optimum in percentage germination at pH 5.8 when determined by citrate-phosphate buffer (Fig. 14a). The percentage germination at all pHs other than 2.8 did not significantly differ ($P = 0.05$) from the unbuffered controls.

Discussion

The percentage germination of V.fungicola conidia in the presence

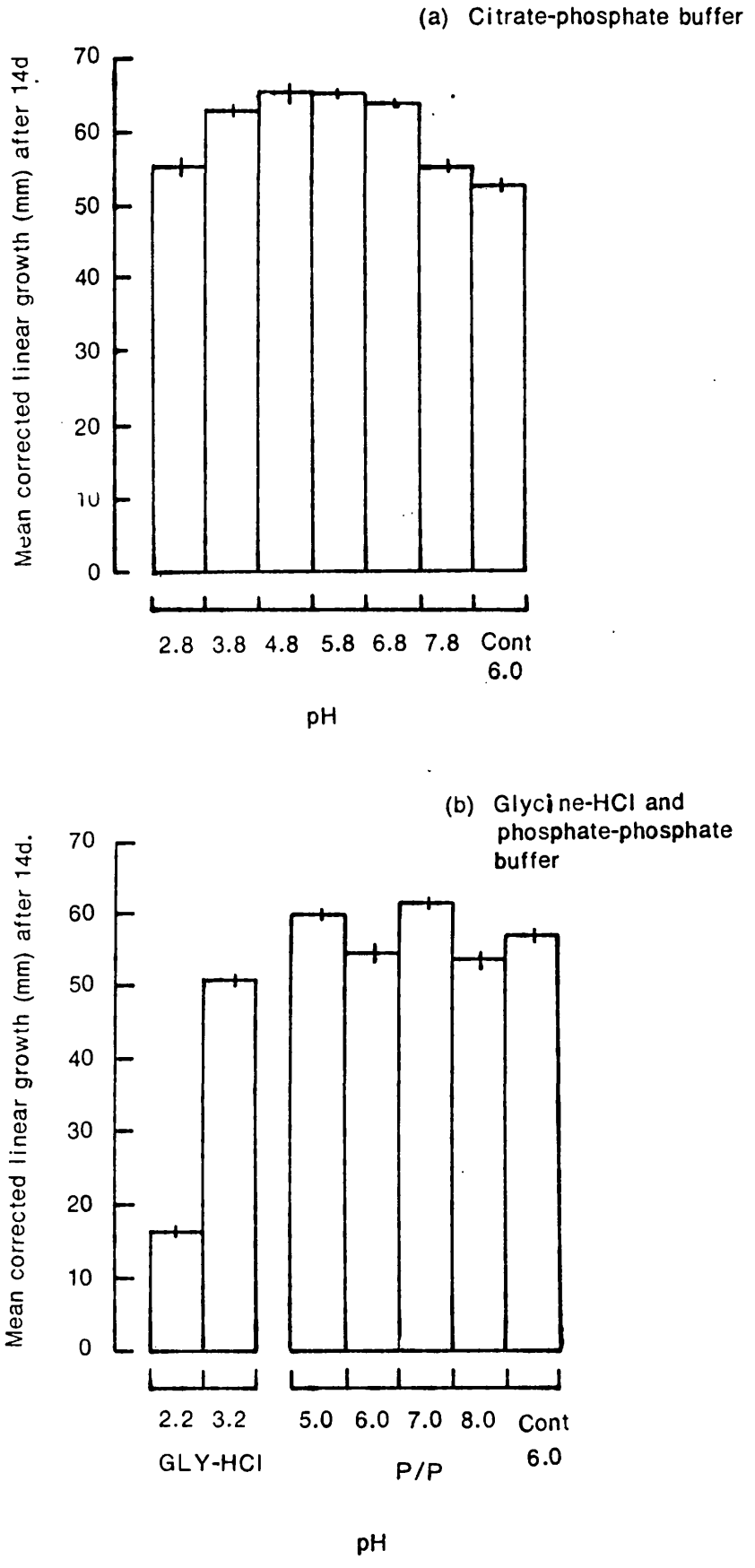

Fig.13. Mean corrected linear growth of *Vf.* isolate G3 on buffered Czapek-Dox agar measured after 14 days at 20°C. (a) Citrate-phosphate buffer. (b) Glycine-HCl and phosphate-phosphate buffers. Control media were unbuffered and were approximately pH 6.0

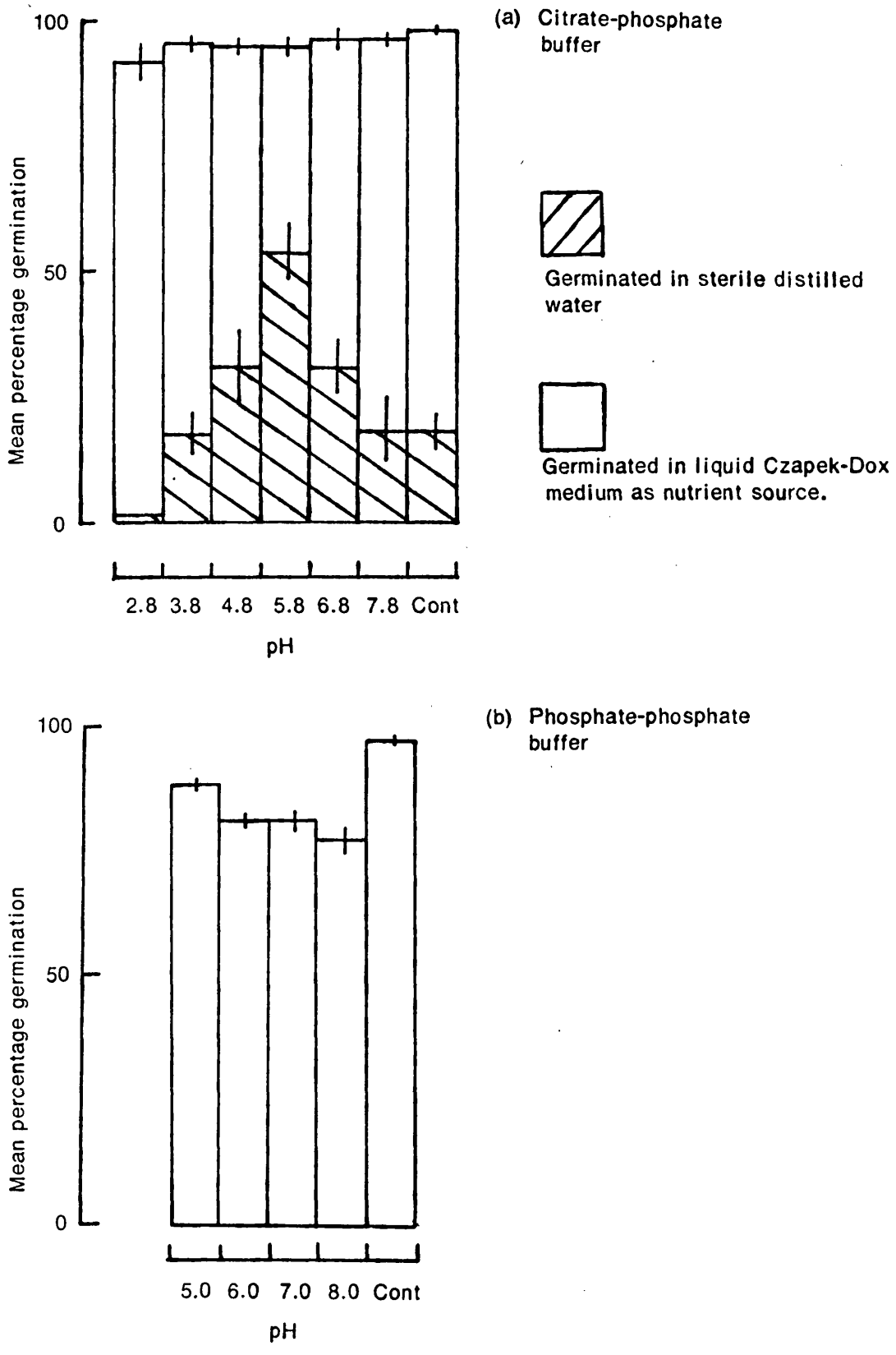

Fig.14. Mean percentage germination of *Vf.* isolate G3 conidia in buffered suspensions measured after 24 hours at 20°C. (a) Citrate-phosphate buffer*in the presence and absence of exogenous nutrients. (b) Phosphate-phosphate buffer. Control suspensions were unbuffered and were approximately pH 6.0

of exogenous nutrients and linear growth of the mycelium were relatively insensitive to a wide range of pH.

In contrast to Treschow's results for the effect of pH on growth in unbuffered liquid culture, there was only a slight optimum for linear growth and this with only one of the buffers.

The greater linear growth at all pHs on media buffered with citrate-phosphate than in the unbuffered control is unlikely to be due to pH alone and may reflect the ability of V.fungicola to use citrate as a carbon source. The concentration of citric acid decreases from 0.9% at pH 2.8 to 0.05% at pH 7.8, which may well be sufficient to influence growth.

When conidia were germinated in citrate-phosphate buffer alone and citric acid was the sole carbon source, germination tended to be greater than in the unbuffered control but this effect was masked when nutrients were not limiting. This again suggests that the citric acid was used as a substrate for growth. The reason for the differences in response of linear growth and germination to citrate-phosphate buffer is not clear but may reflect fundamental differences in the nutrient requirements of the two processes.

There was no obvious trend in linear growth as a response to pH determined by phosphate buffer although there was less growth on the medium buffered at pH 6.0 than on the unbuffered control at the same pH. The phosphate buffer also reduced conidial germination and this suggests that phosphates in concentrations used in the buffers may be inhibitory to V.fungicola. The response of germination and growth of V.fungicola observed with citrate-phosphate buffer may therefore result from an interaction between stimulation by citrate and inhibition by phosphate superimposed on a true pH effect.

The effects of buffers on germination and growth are thus complex

since it is difficult to divorce true pH effects from those of the constituent chemicals. The use of more than one buffer in growth studies however helps to establish the response to pH alone.

DISPERSAL

Using small wind tunnels, Cross (1971) studied the effect of various airspeeds at different r.h. on the dispersal of conidia of V.fungicola from fresh, infected sporophores and agar cultures. Conidia were not dislodged from either source by air flows of up to 11.6 m s^{-1} at 30 to 100% r.h. and Cross concluded that wind dispersal of conidia at airspeeds and humidities that occur in mushroom houses was unlikely.

In the natural environment however, conidia are likely to be exposed to lower r.h. than those occurring in mushroom houses for long periods and this may influence wind dispersal.

Materials and Methods

Wind tunnels were constructed from 20 cm lengths of 1.3 cm internal diameter glass tubing (Zoberi, 1961). The tubes were stoppered at one end and half filled with molten 2% malt agar. After sealing the upper end, the tubes were autoclaved and the agar allowed to set with the tubes held horizontally. Pieces (c. 1 x 0.5 x 0.5 cm) of V.fungicola-infected sporophore bearing sporulating mycelium were placed over glycerol/water mixtures that gave 10, 30, 60, 70, 80, 90 and 100% r.h. at 25°C. After 9, 12, 18 and 25 days' incubation, a piece of tissue from each r.h. treatment and a similarly sized freshly cut piece of a 7 day old colony grown on 1% agar were placed at one end of separate wind tunnels through which air at 40 - 70% r.h. was then drawn at 0.45 m s^{-1} for 3 minutes.

After treatment, the infected sporophore tissue was removed and the V.fungicola mycelium microscopically examined. The tubes were resealed and incubated for 4 days at 20° prior to examination for the presence of V.fungicola colonies along the length of the agar. After 25 days r.h. treatment, mycelium and conidia were transferred from the r.h. treated tissue to fresh sporophores to check for viability.

The release of propagules from agar grown (medium 17Z) cultures of V.fungicola of increasing age (7, 14, 24, 32 day old) was investigated in a similar way to the infected mushroom tissue.

Results and Discussion

Small numbers of viable propagules were only dislodged from sporophore tissue which had been incubated at 10 to 30% r.h. for 12 days. However, propagules were always released from the 7 day old agar grown cultures and the number dislodged increased with the age of the culture up to 24 days (Table 5).

Table 5. The effect of culture age on dispersal of propagules from V.fungicola colonies on medium 17Z

Age of culture (days)	Number of colonies recovered
7	5
14	25
24	continuous streak
32	1

The decrease in colonies recovered after 24 days may be due to loss of viability of the propagules.

Microscopic examination of tissues incubated at r.h. below 70%

showed that many conidial masses remained intact on contact with water which confirms Ware's speculation (Ware, 1933) that drying results in a hardening of the mucilage that surrounds the conidia. These conidial masses, however, were not readily dislodged by air currents.

When mycelium and conidia were removed from tissues kept at less than 90% r.h. for 25 days, and inoculated onto sporophore pilei, brown lesions were produced after 24 h incubation in humid chambers. Above 90% r.h. the mushroom tissue had undergone bacterial decomposition. Lesion development was more severe with mycelium and conidia from the low r.h. treated tissue which again suggested that the speed of drying may have affected survival. That mycelium and conidia remained viable after 25 days incubation at 70 to 90% r.h. (at which conidia and germ tubes lost viability most readily) suggested either that mycelium was resistant to desiccation or that in situ the conidia were protected from drying, possibly by the enveloping mucilage.

The results confirm Cross's view that wind dispersal of V.fungicola conidia from infected tissue is uncommon and show that it is little affected by previous r.h. treatment. In view of this, the consistent recovery of colonies from the agar cultures was surprising and may reflect the particular cultural conditions or the peculiarities of the single isolate used.

CHAPTER 4

PATHOGENESIS

The interaction of V.fungicola with vegetative mushroom hyphae

Cross (1971) demonstrated a close association, in casing, between V.fungicola germ tubes and mushroom hyphae in which the mushroom hyphae appeared to remain undamaged. This association was further investigated in this work by allowing germ tubes and hyphae of V.fungicola to interact with A.bisporus hyphae on both nutrient media and on a glass surface. The response of V.fungicola to two other fungi under similar conditions was also examined.

Cross also suggested that nutrients exuded from mushroom hyphae overcome fungistasis of V.fungicola in casing. A range of concentrations of amino acids and sugars was therefore tested for their ability to stimulate germination of conidia under conditions of simulated fungistasis.

Materials and Methods

The interaction of V.fungicola and A.bisporus on agar was examined by placing 4 x 4 mm pieces from 7 day old colonies of V.fungicola isolates G3, G5, C1 and S1 2 cm from the margins of 10 day old colonies of A.bisporus isolate (Darlington 649) on PDA. The cultures were incubated at 22° and the zone of hyphal interaction between the colonies was examined using a low power objective. Dual cultures of V.fungicola G3 and A.bisporus D649, made on malt, Czapek-Dox and water agar and were similarly examined.

To overcome partially the effect of exogenous nutrients on the interaction between the two fungi, mycelia were allowed to grow together

from 6 mm diameter agar disc inocula placed 1 cm apart on clean, sterile glass microscope slides. These were placed in Petri dishes lined with moist filter paper and incubated at 22°. As the hyphae intermingled, the zone of interaction was examined microscopically and attempts were made to pull apart associated hyphae using a micromanipulator. The interaction between mycelia of V.fungicola and an unidentified mucoraceous fungus isolated from casing was examined under similar conditions.

In another series of observations, A.bisporus and Coprinus lagopus (Fr.) mycelium were similarly grown over sterile microscope slides from disc inocula. After 10 days' incubation at 22° in Petri dishes lined with moist filter paper, the slides were removed and sprayed with a suspension of washed V.fungicola conidia before being returned to the Petri dishes for further incubation. The slides were periodically examined for germination of conidia and development of V.fungicola mycelium.

The ability of V.fungicola hyphae to coil around inert fibres was also examined. Threads of glass wool approximately 10 µm in diameter were washed in distilled water and dry sterilised in glass Petri dishes. When cool, 15 ml of medium 17Z were poured into the dishes, care being taken to ensure that some of the fibres remained above the agar surface. The medium was inoculated with discs of 7 day old colony of V.fungicola and incubated at 22°. The glass fibres in the region of the V.fungicola colonies were periodically examined with a low power objective.

To determine whether the stimulation of germination of V.fungicola conidia by mushroom mycelium could also be brought about by defined chemical solutions, 50 µl drops of washed conidial suspension were pipetted onto clean, sterile cavity slides, together with an equal volume of an autoclaved solution containing a sugar or an amino acid, giving final concentrations of 10, 50 and 500 µg ml^{-1} of the test

material (Table 6).

Table 6. Amino acids and sugars tested for stimualtion of washed V.fungicola conidia.

Chemical	Test concentration $\mu g\ ml^{-1}$		
Asparagine	10	50	500
Tryptophane	10	50	-
Glycine	10	50	-
Cysteine	10	-	-
Casamino acids	10	50	500
Glucose	10	50	500
Fructose	10	50	500
Sucrose	10	50	500
Mannitol	10	50	500
Trehalose	10	-	-
Galactose	10	-	500
Xylose	10	-	500

Sterile distilled water or Czapek-Dox liquid medium were added to controls, the latter to check conidial viability. The percentage germination was measured after 24 h incubation at 20°. The results were subjected to an analysis of variance and LSDs ($P = 0.05$) were calculated.

Results

There was no alteration of the normal colony morphology of either V.fungicola or A.bisporus isolates as they grew together on either PDA

(Plate 9) or on the other media tested. As the colonies met, the marginal hyphae intermingled freely and V.fungicola hyphae occasionally contacted and loosely coiled around A.bisporus hyphae (Plate 10). There was no penetration of, or obvious damage to, the mushroom hyphae.

When mycelia intermingled on a glass surface, however, there was a close association between the hyphae which often grew in contact over large distances (Plate 11 & 12). It was possible to separate the hyphae with a micromanipulator, but this was too crude a method to give a precise idea of the degree of adhesion of the hyphae. Short lateral branches of V.fungicola hyphae occasionally grew towards and contacted A.bisporus hyphae (Plate 13) but in no instances were the mushroom hyphae obviously damaged. V.fungicola hyphae were also observed growing alongside one another (Plate 12).

A similar association was observed between the hyphae of V.fungicola and both the vegetative hyphae and sporangiophores of the mucoraceous fungus (Plates 14 & 15) although again there was no penetration or collapse of cells.

There was little growth of V.fungicola hyphae along the glass fibres, although occasional coiling was observed (Plate 16).

Germ tubes of conidia that had been sprayed onto mycelia of A.bisporus and C.lagopus on glass slides often grew towards and along hyphae of both species (Plates 17 – 21). Although no reliable quantitative estimates of germination were made, it appeared that conidia adjacent to the basidiomycete hyphae germinated more readily and had longer germ tubes than those further away. However, germ tubes of conidia close to a hypha did not invariably grow towards it.

The germination of washed conidia was stimulated by a range of amino acids and sugars, the former having a greater effect at low concentrations (Fig. 15). Relative to the water controls, stimulation was

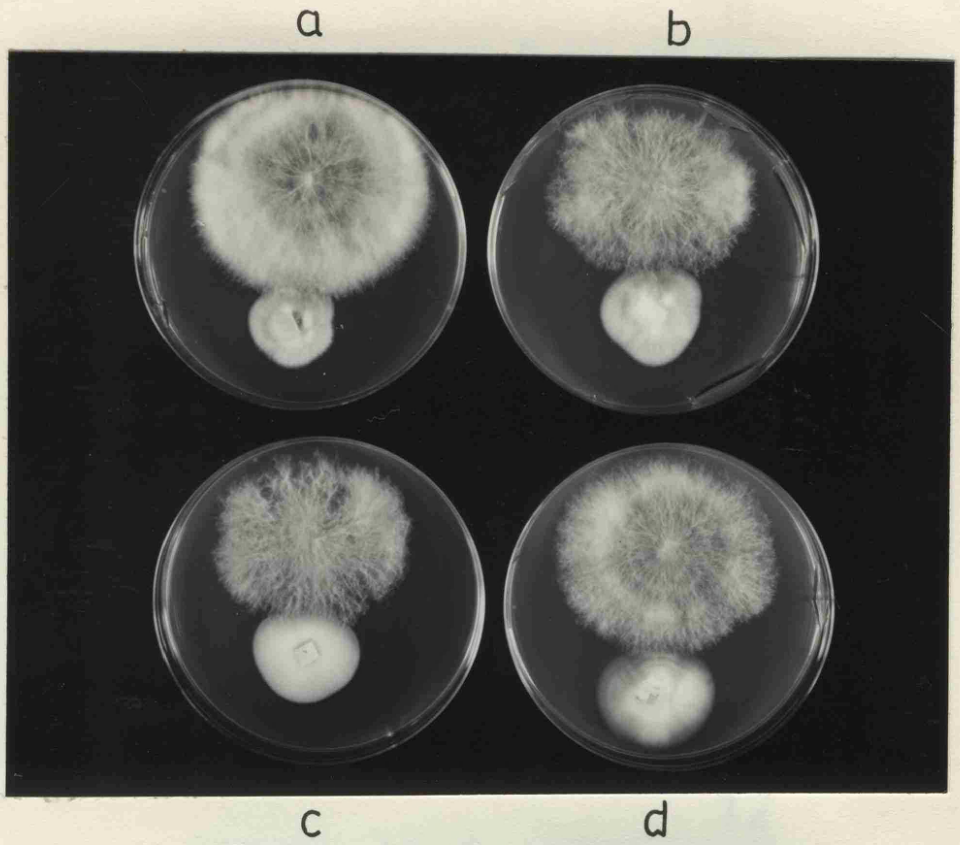

Plate 9 The interaction between 4 isolates of V.fungicola and A.bisporus strain Darlington 649 on PDA. (a) Isolate G3 (b) Isolate S1 (c) Isolate G5 (d) Isolate C1

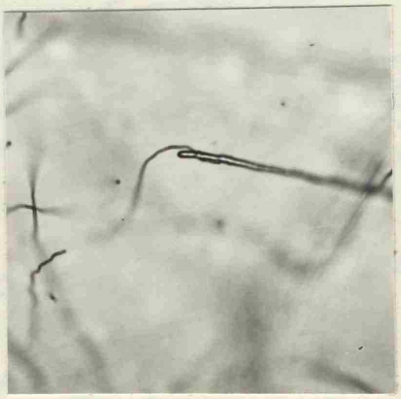

Plate 10 A V.fungicola hypha contacting and loosely coiling around an A.bisporus hypha on a dual culture plate. x 300

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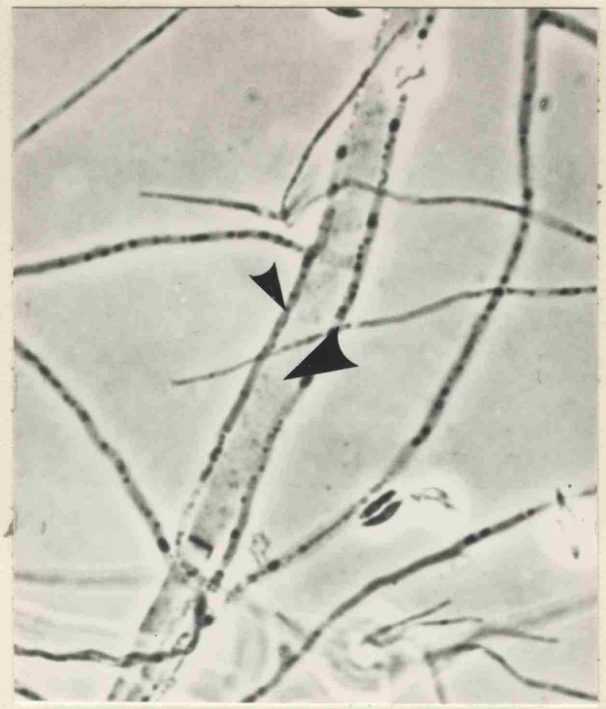

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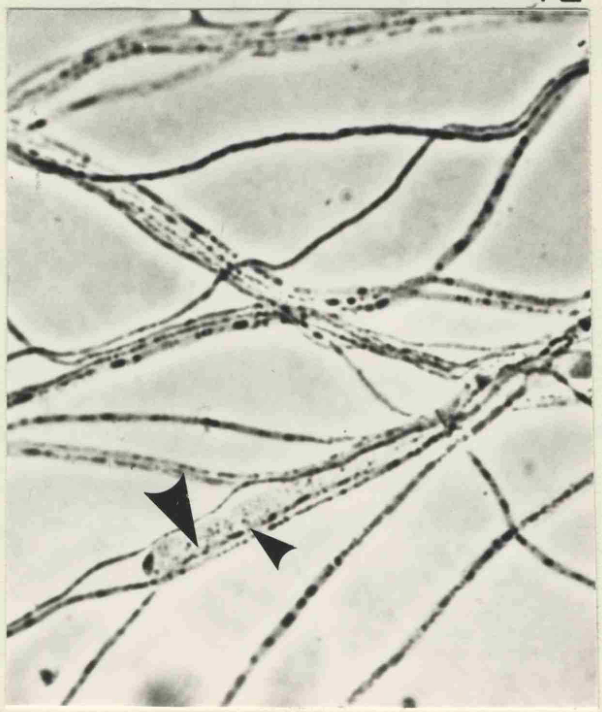


Plates 11 and 12 Hyphal associations between V.fungicola (small arrows) and A.bisporus (large arrows) on glass slides. Plate 12 also shows associations between V.fungicola hyphae. x 700

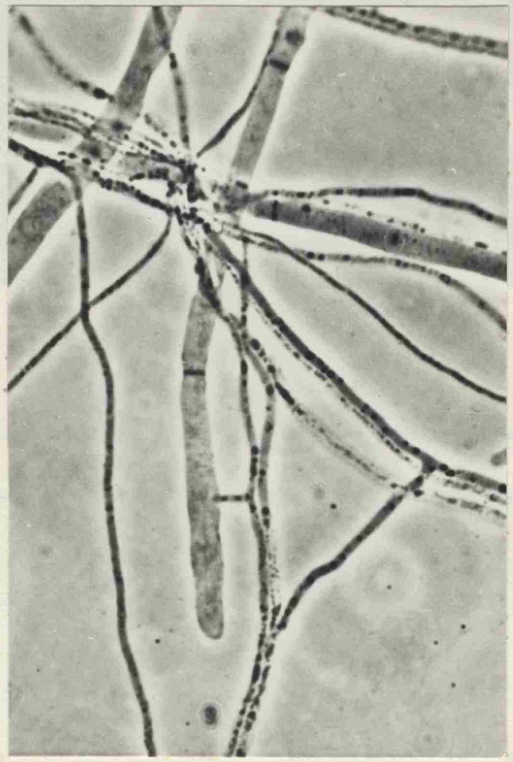


Plate 13 Hyphal association between V.fungicola (small arrow) and A.bisporus (large arrow) on a glass slide. Note lateral hypha from V.fungicola contacting A.bisporus. x 700

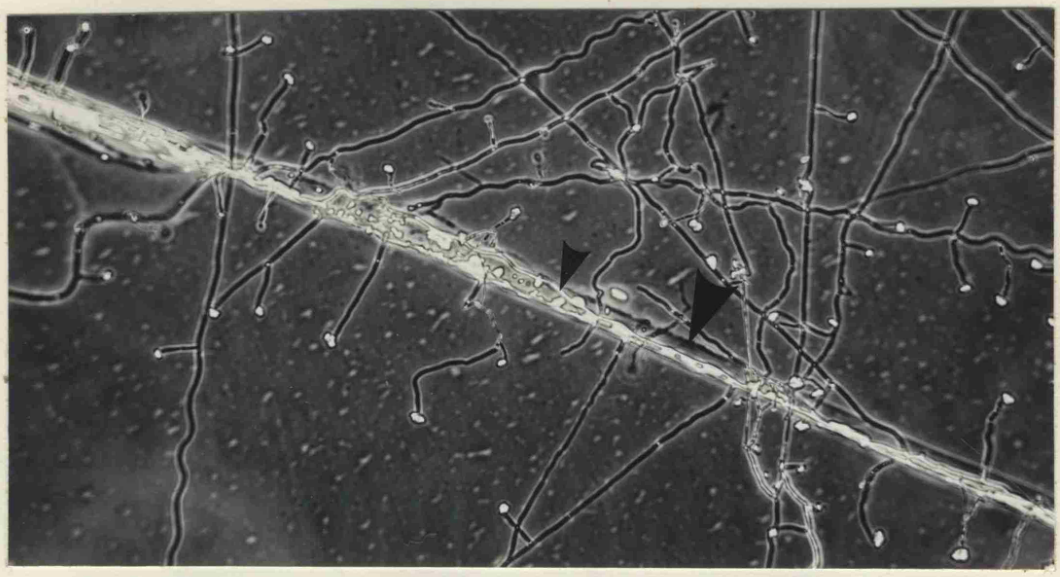


Plate 14 Hyphal association between V.fungicola (small arrow) and a vegetative hypha of an unidentified mucoraceous fungus (large arrow) on a Petri dish base. x 300

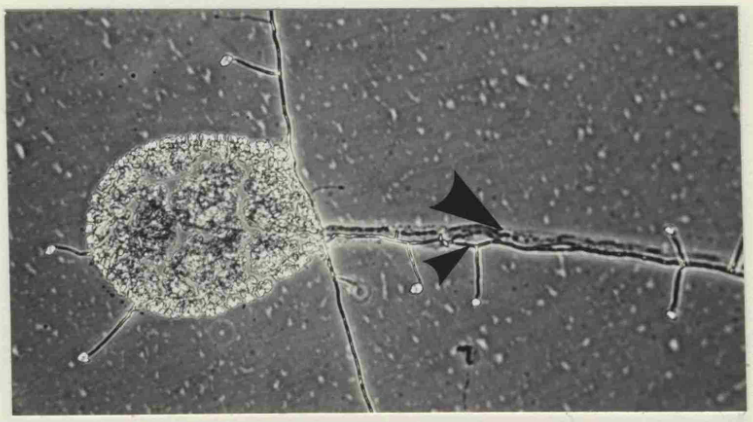


Plate 15 Association between V.fungicola hyphae (small arrow) and a sporangiophore and sporangium of an unidentified mucoraceous fungus (large arrow) on a Petri dish base. x 300

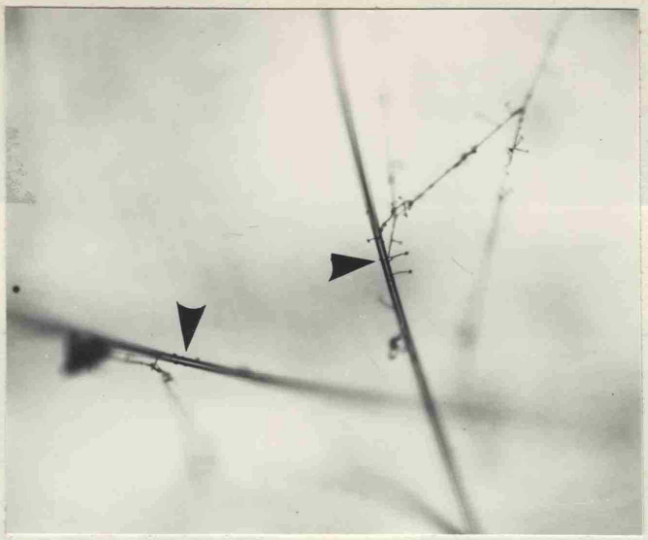
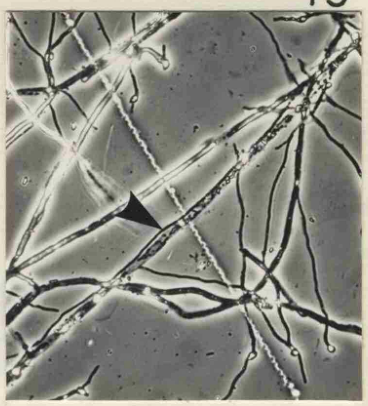


Plate 16 V.fungicola hyphae (arrowed) coiling around glass fibres. x 240

Plate 17 A conidium of V.fungicola germinating in contact with an A.bisporus hypha on a glass slide. x 800



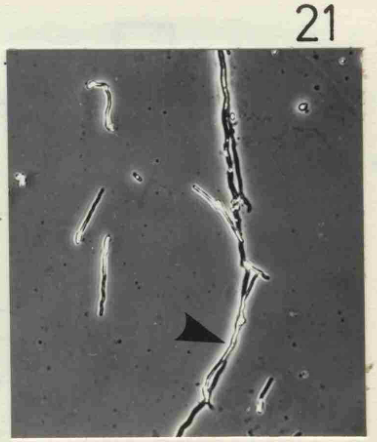


Plate 18 Tropism of V.fungicola germ-tubes towards A.bisporus hyphae on glass slides.

Plate 19 Association between V.fungicola germ-tubes and A.bisporus hyphae on glass slides.

Plate 20 Tropism of V.fungicola germ-tubes towards Coprinus lagopus hyphae on glass slides.

Plate 21 Association between V.fungicola germ-tubes and C.lagopus hyphae on glass slides. Plates 18 - 21 all x 300

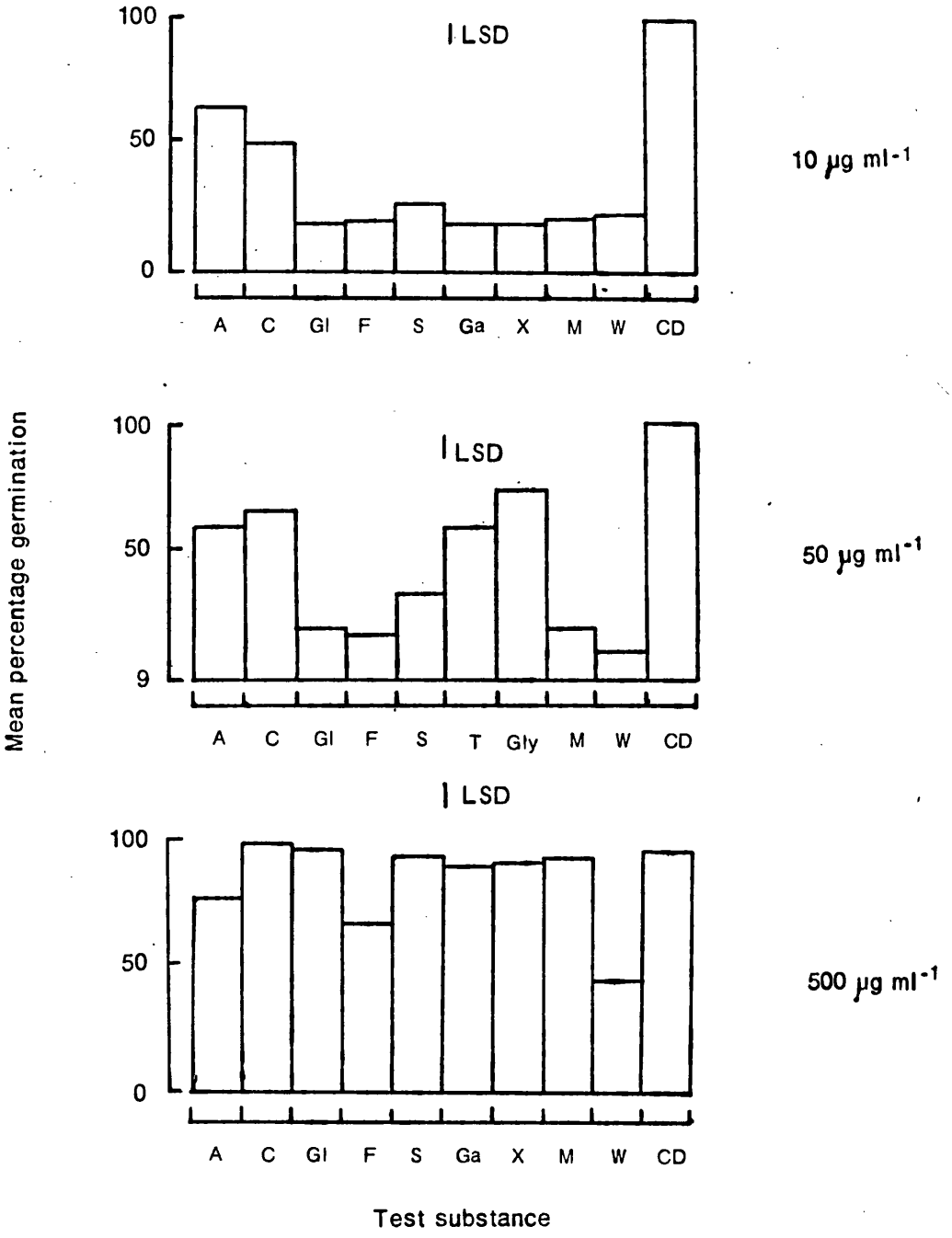


Fig.15. The effect of a range of concentrations of amino acids and sugars on the germination of washed *V. fungicola* conidia.

A = asparagine, C = casamino acids, Gl = glucose, F = fructose, S = sucrose, Ga = galactose, X = xylose, T = tryptophane, Gly = glycine, M = mannitol, W = water control, CD = Czapek-Dox control.

Bars represent LSD of means of 3 replicates (P = 0.05)

independent of concentration of the amino acids (asparagine and casamino acids), whereas the sugars (glucose, fructose, mannitol and sucrose) gave a greater degree of stimulation with increasing concentration (Table 7). The stimulatory effect of amino acids was therefore saturated at 10 μg^{-1}.

Table 7. The effect of increasing concentrations of amino acids and sugars on percentage germination of V.fungicola conidia. Expressed as the difference between percentage germination in the test chemicals and in the water controls. Only amino acids and sugars for which a complete set of data was obtained from tests at every concentration are included in the table.

(Percentage germination in test chemical) - (percentage germination in water control)

Chemical	Concentration $\mu g\ ml^{-1}$		
	10	50	500
Asparagine	43	49	37
Casamino acids	30	56	53
Glucose	-3	9	51
Fructose	-2	7	20
Sucrose	2	29	45
Mannitol	-1	15	49

Discussion

There was no evidence eg. lysis, wavy growth of hyphae, to indicate the production of a diffusable antibiotic by V.fungicola in agar culture. Although the medium used for dual cultures in studies of mycoparasitism can greatly affect the occurrence and degree of

pathogenesis (Butler, 1957; Barnett, 1963), with V.fungicola G3 and A.bisporus D649 no differences in response were noted on the three media used.

Coiling of pathogen hyphae around those of the host is a characteristic of many mycoparasitic relationships (Barnett, 1963), but the coiling of V.fungicola hyphae around A.bisporus was both infrequent and loose and may be partly explained as a thigmotropic response. Hyphal coiling is also usually accompanied by penetration and/or collapse of the host hyphae (Butler, 1957, Rai et al, 1980, Ricard et al, 1974), neither of which occurred in the V.fungicola - A.bisporus interaction.

When nutrient availability was restricted by growing the mycelia over glass slides, V.fungicola exhibited a greater affinity for A.bisporus hyphae. A similar close association between hyphae on glass slides was reported for the mycoparasite Gliocladium roseum and its host Botrytis allii by Walker and Maude (1975). The short lateral branches produced by G.roseum were similar to those produced by V.fungicola although in the former they appeared to bear appressoria and to be associated with penetration. A close examination of the A.bisporus hyphae revealed no apparent damage, although the intimate association of the hyphae suggests that transfer of nutrients may have occurred. However, the mushroom mycelium may not have been the sole source of nutrients for V.fungicola under these conditions, as nutrients could have been translocated from the inoculum disc.

When washed conidia were sprayed onto mushroom mycelia on glass slides, the mushroom hyphae represented the sole source of nutrients for the development of the pathogen. Both germination and germ tube growth appeared to be stimulated in the presence of A.bisporus mycelium and as with mature hyphae, germ tubes grew alongside mushroom hyphae for considerable distances. Cross (1972) observed this phenomenon on glass slides buried in spawn run casing and also reported sporulation of the

pathogen in association with mushroom hyphae. Thus, the ability of V.fungicola to colonise the mushroom 'hyphosphere' may serve to increase inoculum in casing, as well as representing a means by which infection of sporophore primordia may be brought about. A.bisporus was not the only fungus found to stimulate germination and to form hyphal associations with V.fungicola. Similar responses were obtained with C.lagopus and the mucoraceous fungus in vitro, as well as with a Coprinus sp. occurring as a casing contaminant in a separate experiment. Barron and Fletcher (1970) reported that young sporangiophores of Rhopalomyces elegans and several other members of the Mucorales were attacked and internally colonised by both V.fungicola and V.psalliotae. The stimulatory effect of various amino acids and sugars on germination of V.fungicola conidia is further evidence to suggest that the reactions observed between V.fungicola and A.bisporus are non-specific. However, the dense colonisation of casing by A.bisporus makes it likely that the majority of conidia within casing will be stimulated to germinate by and make contact with Agaricus hyphae.

The mechanism by which V.fungicola obtains nutrients from vegetative mushroom mycelium is not clear. After hyphal contact has been established, V.fungicola may either passively absorb exuded nutrients or may actively promote the leakage of nutrients from mushroom hyphae. If the colonisation of the hyphosphere is associated with stimulation of the release of nutrients from host hyphae the mechanism involved must be delicately balanced, since the mushroom hyphae apparently remain healthy. According to the classifications of Cooke (1976) and Rudakov (1978) this would constitute a biotrophic mode of nutrition characteristic of highly specialised mycoparasitic interactions and quite distinct from the necrotrophic parasitism exhibited on infection of mature sporophores.

Whether the ability to colonise the hyphosphere of potential host fungi is peculiar to V.fungicola or other mycoparasites is not known, but since the initial contact between host and pathogen is likely to influence and perhaps determine the course of pathogenesis, the ability to exploit this habitat may be an important attribute of mycoparasitic fungi.

Under the conditions of these investigations, C.lagopus and the Mucor isolate may be viewed as host fungi since it is likely that V.fungicola derived nutrients from them whilst conferring no benefit in return (C.M.I., 1973). It is uncertain whether these fungi would be able to act as hosts under less artificial conditions and whether V.fungicola could cause recognisable symptoms of disease. The ability of particular fungus to act as a host therefore depends on the conditions under which the observations were made.

Infection processes and growth within sporophore tissue

1. Observations

The infection process and growth of the pathogen within mature sporophore tissues were studied, using light, scanning and transmission electron microscopy. Mushroom tissues were examined at different intervals after inoculation and cap lesions were compared with healthy tissue.

Materials and Methods

Surface layers were peeled from healthy sporophores at growth stage 4 (page 19) and were placed, surface upwards, on flamed glass slides in Petri dishes lined with moist filter paper. The tissue strips (c. 1.0 x 0.5 cm) were inoculated with 50 µl of a conidial suspension (2×10^5 conidia ml^{-1}) and incubated at 20°C. After 6, 20, 24 and 42 h, tissue strips were stained with cotton blue in lactophenol and were

microscopically examined.

Segments of lesions were cut from pileal tissue 24 h after inoculation with a V.fungicola conidial suspension (2×10^5 ml^{-1}) and were prepared for scanning electron microscopy as described in the General Methods.

Thin (1 μm) and ultrathin (80 - 100 nm) sections of diseased and healthy tissue embedded in epoxy resin were prepared and examined as described in the General Methods.

Results

The infection process

Conidia on the strips of mushroom tissue had germinated within 16 h and after 20 h deeply stained germtubes were observed running parallel to mushroom hyphae in a similar way to that already described for the interaction of hyphae on glass slides. Many bacteria (Plate 22) were associated with the infection drops and the growth and sporulation of V.fungicola on the strips was limited.

Parallel alignment of V.fungicola and A.bisporus hyphae was also observed in surface lesions examined with the SEM (Plate 23). A fibrillar material was often deposited between host and pathogen hyphae (Plate 24) and A.bisporus hyphae frequently (but not invariably) underwent localised collapse at the point of contact with a V.fungicola hypha (Plates 25 & 26).

Growth of the pathogen within mushroom tissue

A transverse section (TS) of healthy sporophore tissue is shown in Plate 27. The cells are characteristically balloon like and have a large vacuole. Plates 28 - 30 show the progressive collapse of mushroom cells within a 24 h lesion. The mushroom cells first become irregular in

outline, especially where closely associated with V.fungicola hyphae (Plate 28), then lose ridgidity and become flattened (Plate 29) and, at the surface of the lesion, collapse completely (Plate 30). Most of the V.fungicola hyphae were intercellular although occasional intracellular hyphae were observed.

When examined with the TEM, transverse sections of V.fungicola hyphae were readily distinguishable from A.bisporus by their smaller diameter and dense contents (Plates 31 & 32). The cell walls of the two fungi were also distinct, those of the mushroom being thicker and without the electron dense outer layer characteristic of V.fungicola hyphal walls (Plates 33 & 34). In transverse section, V.fungicola cell walls frequently appear to be composed of two electron dense bands separated by an electron translucent region (Plate 34).

The degeneration of mushroom cells, shown by light microscopy, was observed in greater detail by TEM. In infected tissue the mushroom cell walls became indistinct, especially when in close contact with V.fungicola hyphae (Plates 35 & 36) whilst V.fungicola cell walls frequently became sinuous (Plates 37 & 38). The regions of wall contact between the host and pathogen are not obviously differentiated apart from occasional electron dense deposits (Plate 36). The loss of wall substance is especially noticeable in Plate 38 where all that remains of the outer wall is a fibrillar matrix. Digestion of the cell wall is also apparent in Plates 39 and 40 in which a V.fungicola hypha is growing within a mushroom cell wall.

Discussion

The close association between V.fungicola and A.bisporus hyphae on the sporophore surface, similar to that observed on glass slides, suggests that nutrients are exuded from the surface hyphae which influence the development of V.fungicola. Both Cross (1971) and

Plate 22 The surface of a mushroom sporophore below a V.fungicola infection drop 24 h after inoculation. The V.fungicola conidia have germinated and germ tubes penetrated the body of the sporophore. Note the large numbers of bacteria on the sporophore surface. x 2000

Plate 23 Hyphae of V.fungicola (arrow) running parallel to the thicker hyphae of A.bisporus on a sporophore surface 24 h after inoculation. x 5 000

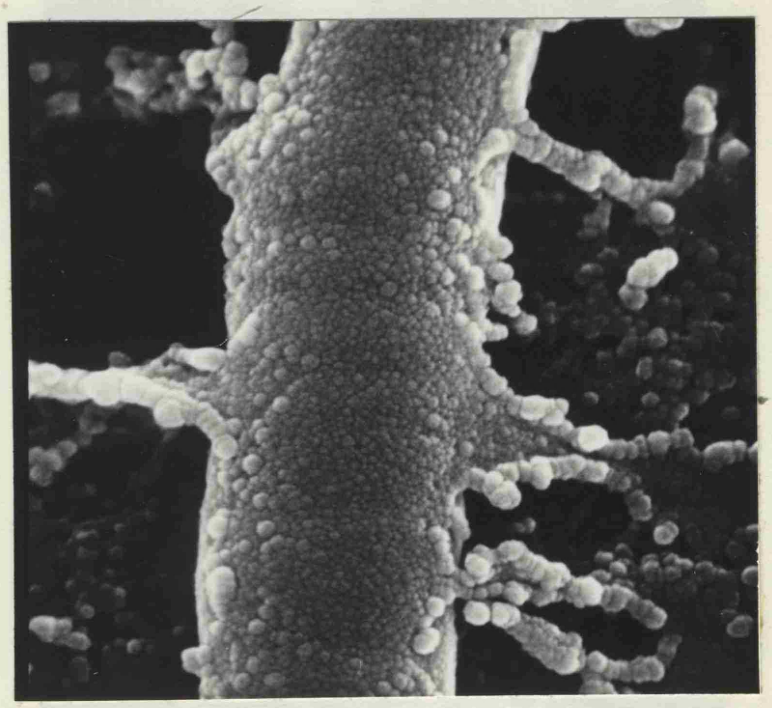
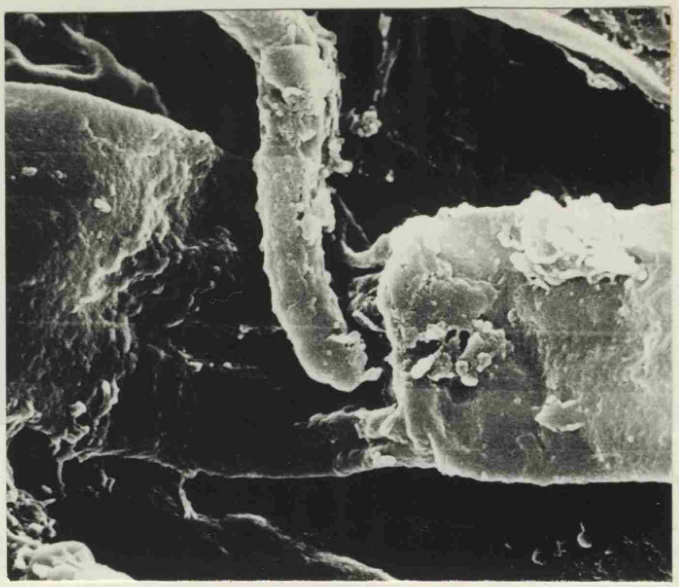
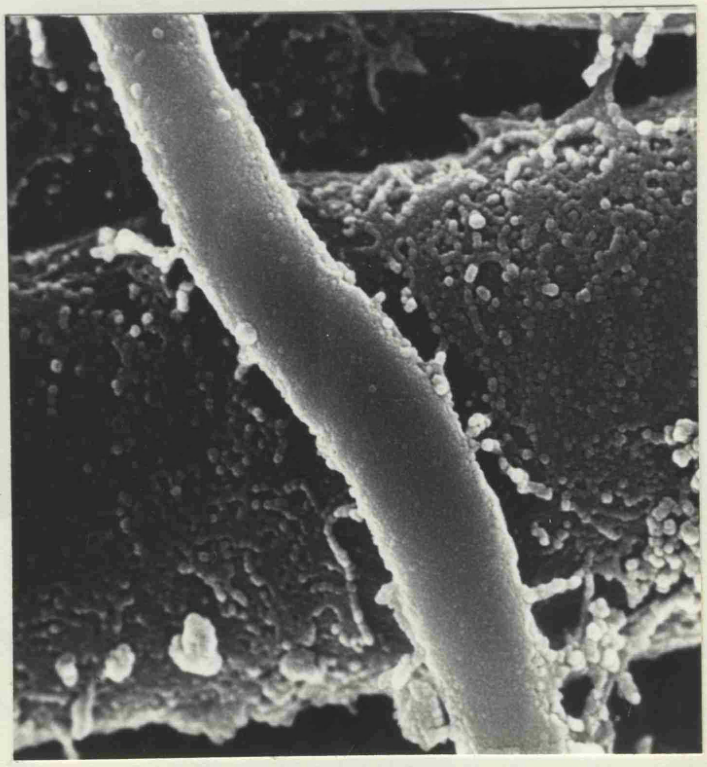


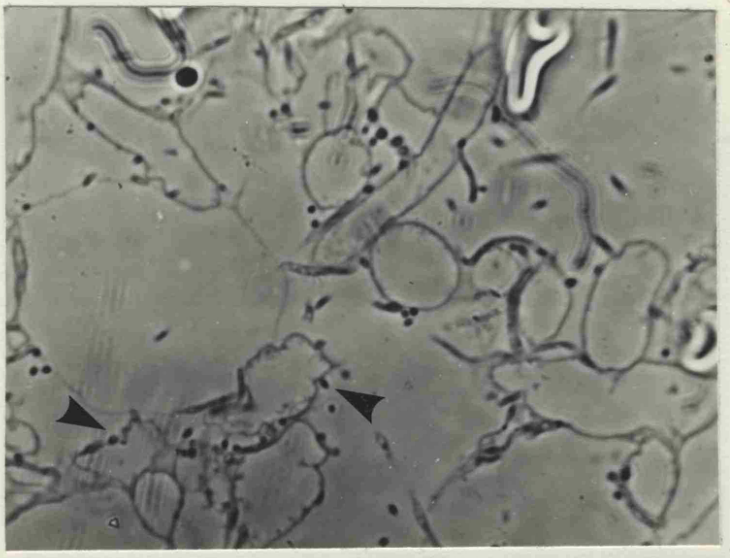
Plate 24 Detail of Plate 23 showing the fibrillar material deposited between V.fungicola and A.bisporus hyphae. x 40 000





Plates 25 and 26 The localised collapse of cap surface of A.bisporus hyphae when contacted by V.fungicola. x 8 000 and 15 000

Plate 27 TS of a healthy mushroom cap tissue. The cells are characteristically balloon shaped and have a large vacuole. x 400



Plates 28 - 30 Showing the progressive collapse of mushroom cells within a 24 h V.fungicola lesion. The lesion extended to a depth of c.2 mm below the cap surface and at the surface bore sporulating V.fungicola mycelium. All x 400

Plate 28 The mushroom cell walls have become irregular in outline, especially when closely associated with V.fungicola hyphae (arrow) but still retain their characteristic shape.

Plate 29 The mushroom cells have become distorted and flattened.

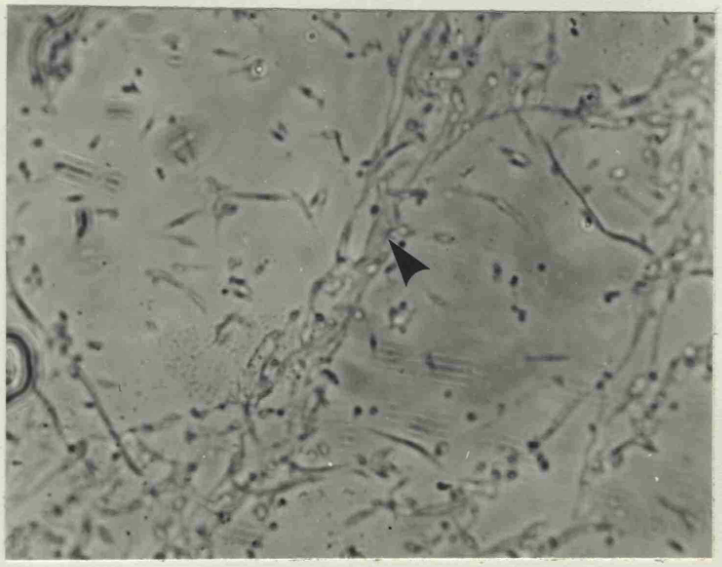


Plate 30 At the surface of the lesion the mushroom cells have collapsed completely and form a dense matrix of cell wall material (arrow) permeated by V.fungicola hyphae.

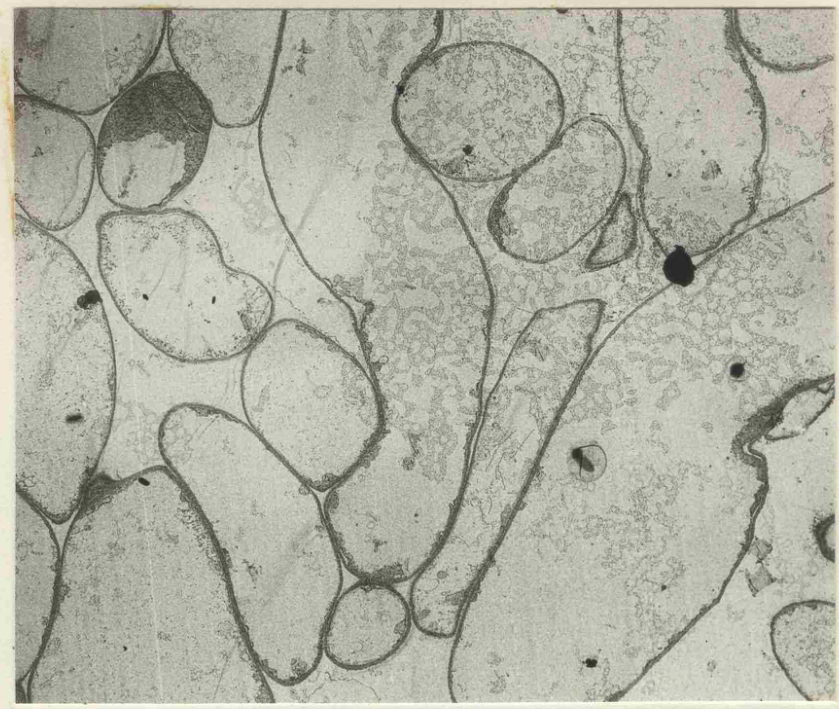


Plate 31 TS of healthy mushroom cap tissue. x 1 600

Plate 32 TS of cap tissue through a V.fungicola lesion showing V.fungicola mycelium. x 1 400

Plate 33 TS healthy mushroom cell wall. x 70 000

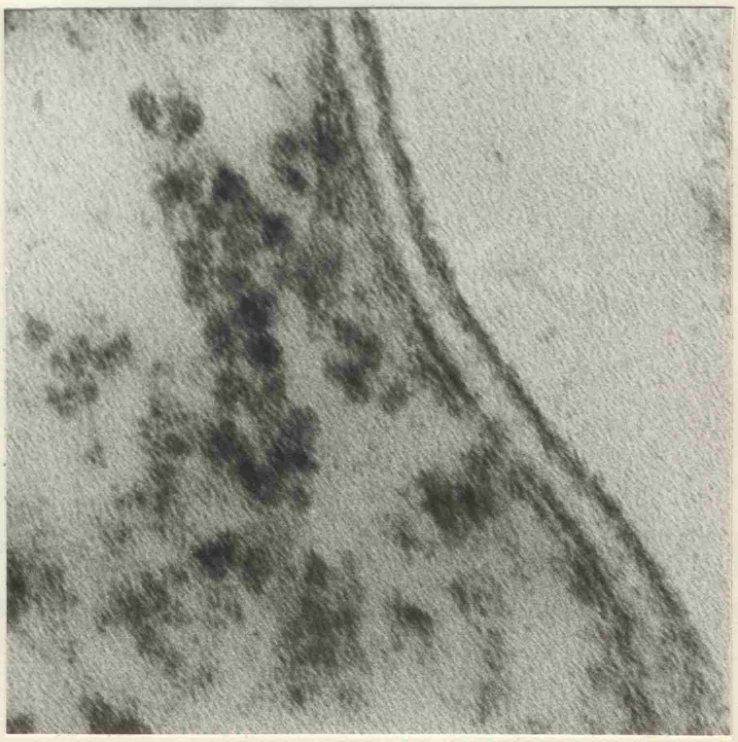


Plate 34 TS V.fungicola cell wall. x 225 000

Plate 35 LS of a V.fungicola hypha growing between two mushroom cells. Note the double wall structure of the V.fungicola cell and electron dense deposit in the mushroom cell on the left. x 14 000

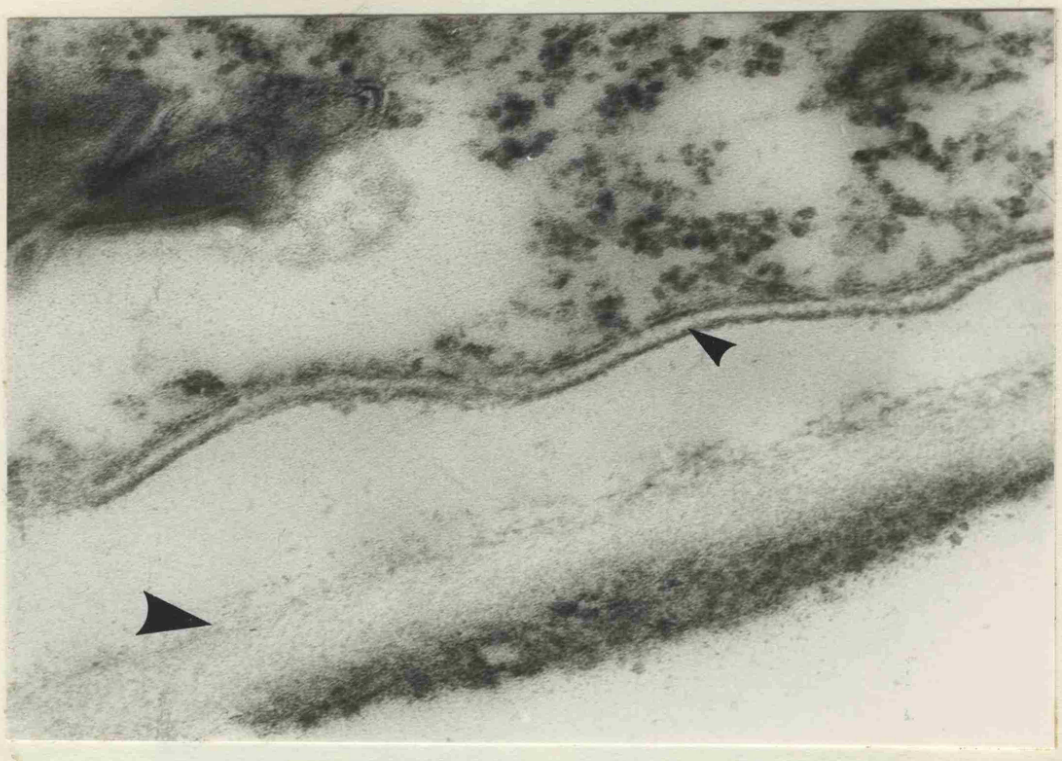


Plate 36 Detail of Plate 35 showing the indistinct structure of the mushroom cell wall, particularly in its outer layers (large arrow), and the electron dense wall deposit. Note also the dense concentration of ribosomes in the V.fungicola cell and the sinusoidal, double layered wall (small arrow). x 100 000

Plate 37 LS of V.fungicola hypha contacting a mushroom hypha.

x 12 000

Plate 38 Detail of Plate 37 showing the indistinct nature of the outer regions of the mushroom cell wall (arrow). Although the cell walls appear to merge in this micrograph, this is an artefact due to the obliqueness of the section. x 69 000

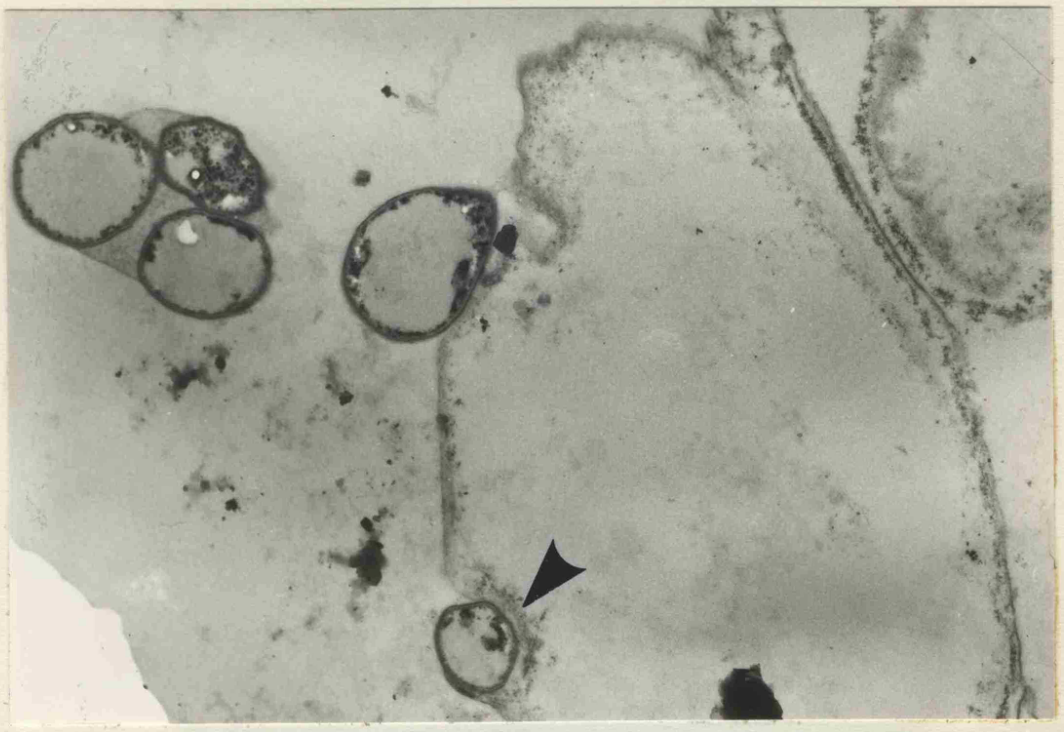


Plate 39 TS of V.fungicola hyphae within mushroom tissue in close association with a mushroom cell. The lower Verticillium hypha appears to be growing into the mushroom cell wall (arrow).
x 12 000

Plate 40 Detail of Plate 39 showing V.fungicola hypha growing into the mushroom cell wall which has again become indistinct. x 50 000

Vincent-Davies (1973) showed that exudates from sporophores stimulated the germination of V.fungicola conidia in vitro. V.fungicola germ tubes did not, however, remain solely associated with surface hyphae but penetrated into the body of the sporophore which is composed of an open matrix of hyphae.

The macroscopic symptoms of infection of mature sporophores imply that either toxins and/or enzymes are involved in pathogenesis. The microscopic symptoms of infection as revealed by the scanning and transmission electron micrographs show that the collapse of mushroom cells is caused by the digestion of the cell wall. Wall dissolution is probably due to the action of extracellular enzymes since the walls of cells not in intimate contact with V.fungicola hyphae also lose integrity. V.fungicola hyphae were only present in cells that had partially collapsed and which may have been breached elsewhere; in no instance was the direct penetration of mushroom cells observed.

A fibrillar deposit between host and pathogen hyphae has also been observed in other mycoparasitic interactions involving Verticillium spp. eg. infections of Alternaria brassicae by Nectria inventa (Verticillium lateritium) (Tsuneda and Skoropad, 1977) and Hemileia vastatrix by Verticillium lecanii (Locci et al, 1971) and in various fungus-higher plant interactions (Bracker and Littlefield, 1973). Its function is thought to be adhesive.

2. Mechanisms of pathogenesis

(i) Symptoms produced on infection of mature sporophores

Attempts were made to reproduce the symptoms of infection of mature sporophores using cell free culture filtrates and mycelial extracts of V.fungicola and to elucidate the nature of the degradative principle involved.

Materials and Methods

a) Preparation of the extracts

Culture filtrate.V.fungicola G3 was grown in static culture in 250 ml flasks at 20°. The culture medium used was 2% w/v mushroom flour in distilled water. The mycelia were harvested by filtration through Whatman No 1 filter paper after 7 days and the filtrate passed through a washed Carlson Ford HP/EKS grade filter pad under reduced pressure. The filtrates were sterilised by filtration through a 0.22 µm Millipore membrane.

Mycelial extract. Mycelial mats grown as described above were washed with distilled water, blotted dry, weighed and homogenised with distilled water (c. 1:5 w/v) in a Silversen blender at top speed for 3 minutes. The homogenates were subjected to ultrasonic disintegration in an ice bath for 3 min, after which over 90% of the hyphal fragments appeared empty when viewed under phase contrast. The homogenates were filtered through Whatman No 1 filter paper, a Carlson Ford HP/EKS grade filter pad and the filtrates were sterilised by passage through a 0.22 µm Millipore membrane.

Infected and healthy mushroom tissue extracts. Detached sporophore caps were painted with a concentrated suspension of V.fungicola conidia using a camel hair brush and were incubated in humid chambers at room temperature for 3 days, by which time the tissues were well colonised. Control sporophores were painted with sterile distilled water. After 3 days, the surface layers from inoculated sporophores were peeled off and were ground with acid washed sand and homogenised with distilled water (c. 1:2 w/v) in a Silversen blender at top speed for 3 min. The extracts were then filtered and sterilised as described for the mycelial extract.

In later experiments, diseased and healthy tissue extracts were either boiled or autoclaved before application to the sporophores.

Other extract preparations were concentrated using an Amicon P10 molecular filter and both high and low molecular weight fractions were tested for their ability to cause tissue collapse.

Drops (c. 50 µl) of the three extracts were pipetted onto the surface of 8 detached sporophores, four of which had been peeled to remove the surface microflora. Control treatments consisted of drops of uninoculated culture medium in the culture filtrate test, drops of sterile distilled water in the mycelial extract test and drops of uninoculated tissue extract in the tissue extract test. The sporophores were incubated at room temperature in humid chambers and were periodically examined for signs of tissue collapse below the drops.

A crude assay system for the degradative principle was established using ten-fold serial dilutions of the extracts in sterile distilled water. Drops of each dilution were pipetted onto peeled and unpeeled sporophore caps which were incubated as above.

Culture filtrate, cap surface tissue extracts and extracts of sclerodermoid and healthy sporophores prepared in a similar way to the cap surface tissue extracts were tested for the presence of substances with antifungal activity (Ebben & Spencer, 1973). Drops of the filtrates and extracts (20 - 50 µl) were spotted or streaked onto strips of Whatman 3MM chromatography paper. Controls were streaked with either uninoculated culture filtrate or extracts of healthy cap surface tissue or sporophores. The strips were sprayed with a concentrated suspension of conidia of Fulvia fulva (Cooke)Ciferri in half strength liquid Czapek-Dox medium. The strips were hung in sealed beakers to maintain a high humidity and were periodically examined to determine if development of F.fulva had been inhibited by any of the extracts.

Results

Only extracts of inoculated sporophores consistently caused

sinking of sporophore tissue beneath the test drop. The tissue collapse was more apparent on peeled than unpeeled sporophores and took several days to become pronounced (Plate 41c). The active principle was destroyed by both boiling and autoclaving (Plates 41a & 41b). The activity of the original extract was reduced by dilution, being slight in a 100 fold dilution and completely lost at 1000 fold dilution. Molecular filtration of the extract showed that the activity was associated with the high molecular weight fraction (>10,000) and when this was concentrated approximately 10 fold, the collapse of sporophore tissue occurred within 16 h of application.

There was no evidence of antifungal compounds in V.fungicola culture filtrates, cap surface tissue or sclerodermoid sporophore extracts. The test fungus F.fulva grew more densely on the streaks than on the surrounding areas, probably because of nutrients present in the extracts.

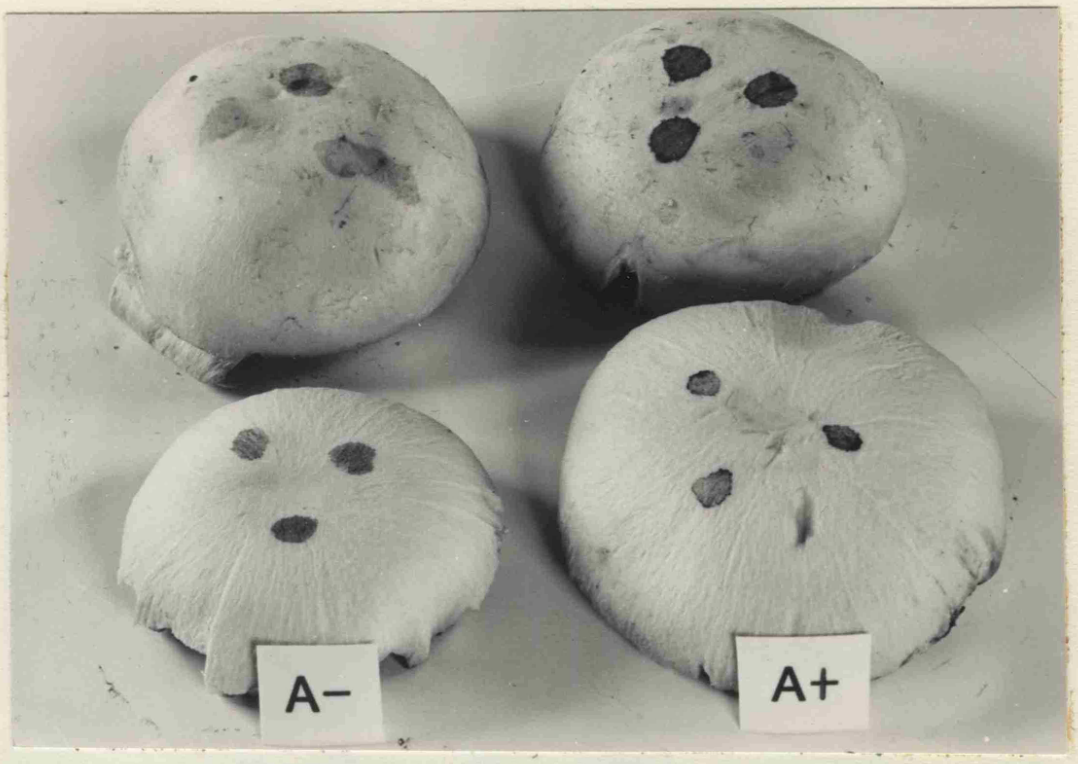
Discussion

The slow activity, thermolability and relatively high molecular weight of the degradative principle in the diseased tissue extract, together with the microscopic observations, strongly suggest that tissue collapse is due to the action of extracellular enzyme(s) on mushroom cell walls. The enzyme(s) were stable at room temperature but were not induced in liquid culture with dried mushroom flour as the sole carbon and nitrogen source. The presence of degradative activity in the tissue extract alone suggests that a close physical relationship between the pathogen and living host tissue is necessary for enzyme production.

Degradation of mushroom cell walls and their constituent polymers in vitro.

Mushroom cell wall structure

The composition and architecture of the cell walls of A.bisporus



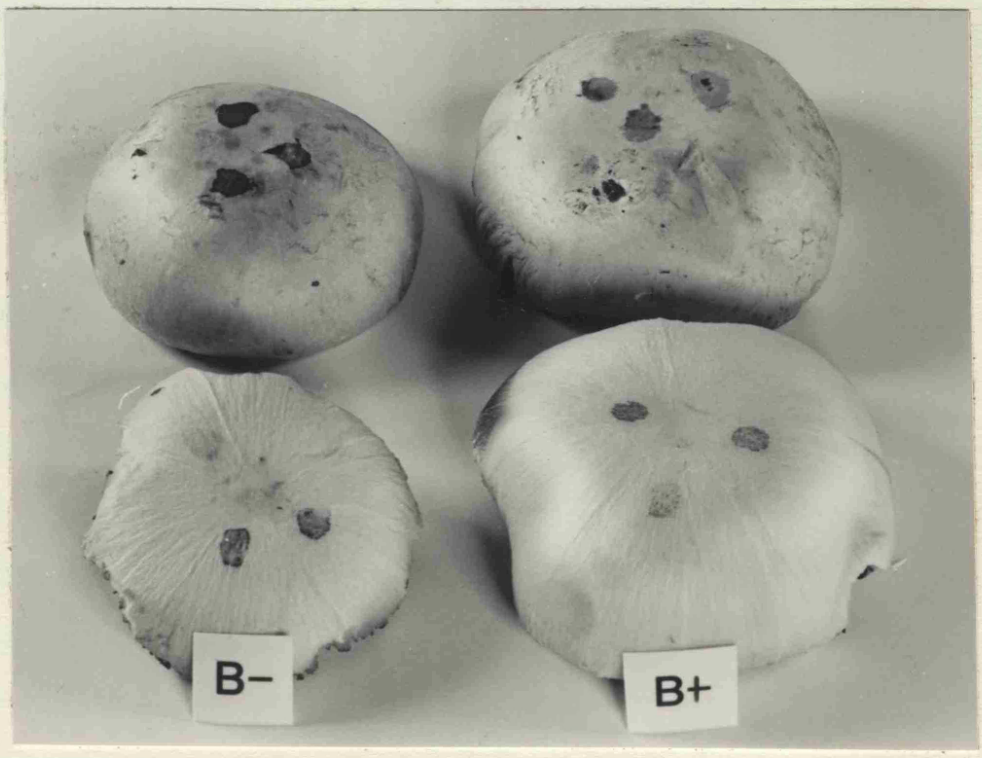


Plate 41 The degradation of sporophore tissue by a cell-free extract of V.fungicola infected sporophore tissue.

For explanation, see following page.

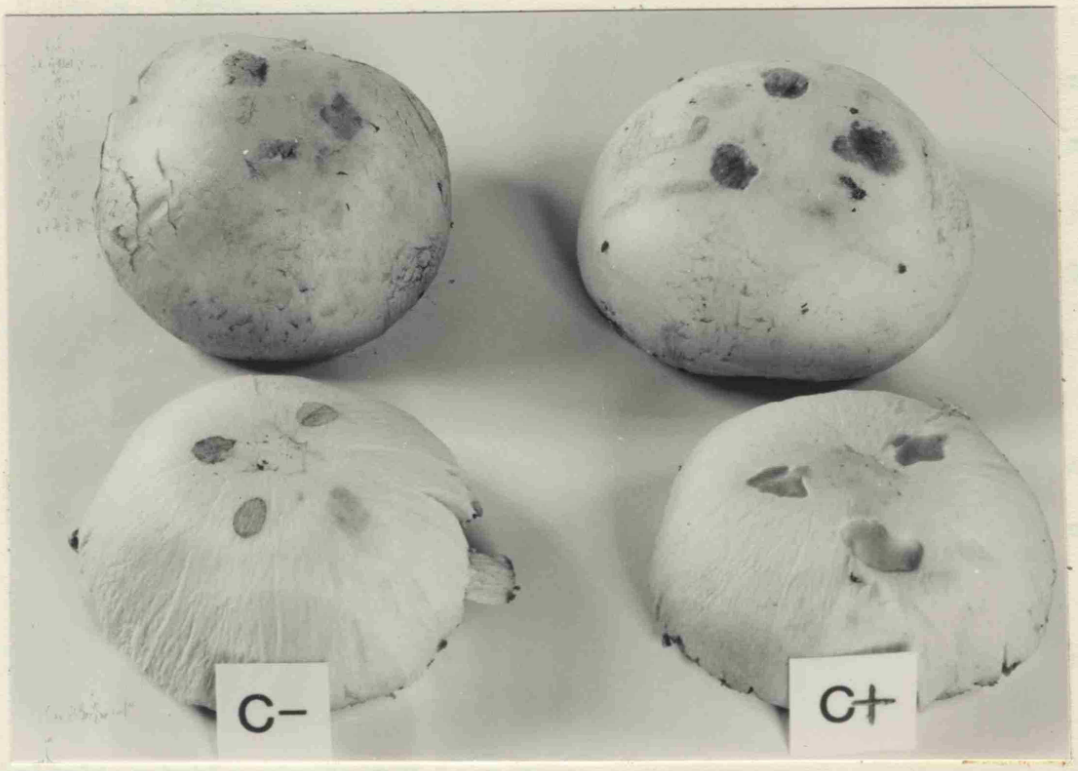


Plate 41 continued

A+ autoclaved extract of infected tissue.

A- autoclaved extract of healthy tissue.

B+ boiled extract of infected tissue.

B- boiled extract of healthy tissue.

C+ untreated extract of infected tissue. Note the collapse of tissue in both the peeled and unpeeled sporophores.

C- untreated extract of healthy tissue.

and other fungi has been elucidated by means of the sequential extraction of the constituent polymers with acids, alkalis and enzyme preparations, coupled with electron microscopy and X-ray diffraction analysis of the extracted polymers (Michalenko et al, 1976; Vincent-Davies, 1973; Wessels, 1965).

A.bisporus cell walls are of the chitin-glucan type according to the classification of Bartniki-Garcia (1968). In this wall type microfibrillar chitin constitutes the innermost layer of the wall and is thought to be the major polymer conferring mechanical strength. The glucans present are of two types. The bulk of the wall thickness in vegetative cells is composed of an alkali in-soluble β 1-3 linked mixed glucan which has a small proportion of β1-6 linkages. This glucan is similar to the R glucan described by Wessels (1965) in the walls of Schizophyllum commune and yeast 'hydroglucan' (Kreger, 1954). It is thought to be widely distributed in fungal cell walls (Rosengurger, 1976). The outermost layer of mushroom cell walls is composed of an alkali soluble mixed α1-3 linked glucan similar to the S glucan of S.commune.

There are differences in the architecture of vegetative and sporophore cell walls. The rapid expansion of cells in sporophores of both S.commune and A.bisporus is accompanied by a decrease in the amount of β1-3, β1-6 glucan which is thought to account for the greater extensibility of the hyphae (Vincent-Davies, 1973). In A.bisporus chitin microfibrils become distributed throughout the wall which may serve to maintain mechanical strength whilst there is little change in the amount of α glucan present.

Other wall constituents include protein and lipids, but whilst the former is widely distributed throughout the wall and appears to be a structural component (Rosenburger, 1976), the nature and function of lipids in the wall are not understood.

The ability of isolates of V.fungicola and several non-pathogenic Verticillium species to degrade a mushroom cell wall preparation and extracted wall polymers was examined using a cleared zone assay.

Materials and Methods

Preparation and fractionation of mushroom cell walls. The methods described in this section were similar to those used by Vincent-Davies (1973).

Cell walls were extracted from 1 kg of stipes of sporophores at growth stage 4. The stipes were extensively homogenised in 700 ml of distilled water using a Silversen blender, followed by centrifugation at 23,000 g for 10 min. The pellets were boiled in 1% sodium dodecyl sulphate for 15 min., centrifugally washed and the cells disrupted in a French press, achieving a high percentage breakage of cells. The walls were washed with 10 l of distilled water and freeze dried.

Glucans were extracted from a 5 g sample of the dried cell wall preparation. The walls were shaken with 5% KOH for 17 h at room temperature, the suspension centrifuged and the supernatant titrated with glacial acetic acid to pH 4.5 The white precipitate formed was sedimented, washed twice with 0.5N acetic acid and four times with distilled water. This precipitate corresponds to the α 1-3 glucan fraction. (Kreger, 1954; Vincent-Davies, 1973).

The insoluble fraction was washed several times with distilled water and was boiled with N HCl for 1 h. The insoluble residues from this extraction were washed and further extracted with 0.75N NaOH at 60° for 30 min. and, after centrifugation at 23,000 g for 10 min., the supernatant was titrated with glacial acetic acid to pH 4.5. A very faint precipitate was formed corresponding to the β1-3, 1-6 glucan ('hydroglucan') fraction (Vincent-Davies, 1973). Unfortunately,

insufficient was recovered to use in a cleared zone assay.

Polymer media and cleared zone assay. The dry cell wall preparation was added to Malca's salt solution (Malca et al, 1966) at 0.3% w/v and the ∝ 1-3 glucan, colloidal chitin(prepared from crab shells)and insoluble casein at 1% w/v. All the media were solidified with 1.5% w/v agar. After boiling, the media were dispensed into test tubes to a depth of 3 cm and autoclaved. To prevent the substrates from sedimenting, the tubes were shaken while the media were rapidly solidified in an ice bath.

The media were inoculated with agar colony discs of isolates of V.fungicola and V.psalliotae and several species of Verticillium not pathogenic to mushrooms and were incubated at 20°. Cleared zone depths were measured after 7 d for casein, 65 d for chitin and 54 d for the ∝ glucan and cell wall preparation.

β glucanase assay. Because little β glucan was recovered from the cell wall fractionation and the only commercially available source of β glucan (laminarin) was soluble and therefore unsuitable for a cleared zone assay, β glucanase activity was therefore estimated as the release of reducing sugars from laminarin by culture filtrates.

Cultures of V.fungicola isolate G3 grown in 2% w/v mushroom flour medium at 20°C were harvested after 7 days. 0.25 ml of the culture filtrate were added to an equal volume of 1% laminarin in 0.1N acetate buffer pH 5.0 in thin walled glass test tubes. The mixture was incubated at 25°C for 1 hour and the reaction terminated by boiling the tubes for 10 minutes. A second set of tubes containing the same reaction mixture were boiled for 10 minutes prior to incubation and constituted the 'boiled controls'. Other controls in which either the substrate or the

culture filtrate were omitted from the reaction mixture (the volume being made up with acetate buffer) were also prepared and were treated in the same way as the experimental tubes. The total reducing sugar concentrations were determined after incubation by the colorimetric method of Nelson(1944) and Somogyi (1945) and the concentrations in the reaction mixture and the boiled controls were compared using Student's t test.

Results

With the ∝ glucan and the cell wall preparation there were no true cleared zones, although the media became differentiated into two regions. In the upper region of both media, the substrates became partially translucent and homogenous, suggesting that limited degradation was taking place.

All five of the isolates of V.fungicola degraded the wall polymers tested in the assays, but there was considerable variation between the isolates in the amount of degradation of the individual polymers (Table 8). There were no consistent qualititive trends in the ability of the different isolates to degrade the polymers. For example, isolate C1 produced a deep cleared zone on chitin but degraded both the cell wall preparation and protein poorly, whilst with isolate G3, the reverse occurred. The significance of the qualititive differences between the isolates is uncertain however, since there were often large differences in the depths of clearing produced in replicate tubes of a single isolate. It should also be borne in mind that an ability to degrade the wall polymers in vitro may not necessarily reflect the situation in vivo.

An isolate of the mushroom pathogen, V.psalliotae showed a similar ability to the V.fungicola isolates in degrading all 4 polymers. With chitin, there was again intraspecific variation in degradative ability.

Table 8. Depths (mm) of cleared zones in mushroom cell wall and wall polymer media. The figures represent the mean of duplicate tubes, except for the ∝ glucan medium, for which only one tube was set up for each isolate.

Isolate	Identity	Chitin (65d)	Casein (7d)	∝glucan* (54d)	Cell walls* (54d)
G3(M)		5.0	13.0	3.0	8.0
G4(M)		6.5	9.0	3.0	6.5
G5(M)	V.fungicola	9.5	14.0	3.0	7.5
C1(M)		10.5	3.5	6.0	3.0
S1(M)		10.0	8.0	5.0	2.5
C9(M)		20.0	16.0	6.0	3.0
P219	V.psalliotae	11.0	ND	ND	ND
P398		10.0	ND	ND	ND
M1(M)		9.5	10.0	4.0	0.0
LL72	V.lamellicola	17.5	19.0	ND	ND
P282		15.0	ND	ND	ND
C3	V.lecanii	ND	ND	5.0	5.0
ST29		ND	ND	4.0	2.0
V1	V.lateritium	ND	ND	0.0	0.0
R18	V.bulbillosum	ND	ND	ND	2.0

ND Not determined

(M) Isolate obtained from mushrooms

* With these two substrates no true cleared zone was formed, although the media became differentiated into 2 regions. In the upper region, the depth of which is recorded in the table, the substrates became partially translucent and the cell wall medium lost its particulate form.

An isolate of Verticillium lamellicola obtained from mushrooms and an isolate of Verticillium lateritium obtained from soil failed to degrade the cell wall preparation. Two isolates of V.lecanii, one from aphids and the other from a spore trap slide, and an isolate of V.bulbillosum from soil caused a degradation similar to that obtained with some of the V.fungicola isolates.

There was no significant β glucanase activity in the V.fungicola culture filtrate (Table 9).

Table 9. β glucanase assay. The release of glucose equivalents from laminarin (β 1-3, glucan) by V.fungicola culture filtrate.

Enzyme activity (mg of glucose equivalents released ml^{-1} of extract h^{-1})			
Culture filtrate*	Boiled controls+	- substrate+ controls	- culture+ filtrate control
47.5^{a}	46.4^{a}	45.6^{a}	7.2

* mean of 6 replicate tubes

\+ mean of duplicate tubes

a not significantly different at $P = 0.05$

Discussion

The V.fungicola isolates were capable of partially degrading mushroom cell walls and their constituent polymers in vitro but there was no evidence for the production of an extracellular β glucanase.

The results confirm and extend those of Vincent-Davies (1973) who demonstrated that a single isolate of V.fungicola could degrade mushroom cell walls, the component polymers tested here and also a mixed β 1-3, 1-6 glucan obtained from yeast cell walls that is thought to be similar to the β glucan found in Agaricus walls. Although the absence of extracellular β 1-3 glucanase activity is therefore surprising, Vincent-Davies observed a similar phenomenon in studies of enzyme production by M.perniciosa; live cultures were able to degrade all the cell wall components tested, but culture filtrates showed little activity against ∝ glucan and chitin. A possible explanation is that only one isolate of V.fungicola was tested for β glucanase activity and a large intraspecific variation in enzyme production was demonstrated in V.fungicola by Trigiano and Fergus (1980) and was also found with the other polymers in this study.

All of the isolates included in these tests were highly pathogenic towards mushrooms but degraded the cell wall preparation to different extents. Although there are likely to be differences in pathogenicity between the V.fungicola isolates the extent of these is unknown and it remains uncertain whether intraspecific variation in pathogenicity may be accounted for by differences in cell wall degrading ability. If the results obtained in these in vitro studies correspond to the degradative abilities of the fungi in vivo, they suggest that pathogenicity at the specific level is not solely determined by the ability to degrade cell walls, since a non-pathogenic species such as V.bulbillosum exhibited a similar degree of degradation to V.fungicola isolate S1.

With other species, eg. V.lateritium, which is capable of parasitising other fungi, non-pathogenicity towards mushrooms coincided with an inability to degrade mushroom cell walls. It is interesting that an isolate of V.lamellicola, a fungus that frequently develops on the surface of cut sporophores in humid conditions but which does not appear to be truly pathogenic (Gandy, 1973), was also unable to degrade the wall preparation. Whilst not considered as a pathogenic species, V.lecanii can, under certain conditions, form lesions on cut sporophores similar to those produced by V.fungicola and both of the isolates tested were capable of some cell wall degradation.

As with the fungal diseases of higher plants, the mechanism of parasitism in mycoparasitic interactions may take various forms which include production of antibiotics, contact parasitism, penetration and production of haustoria and production of enzymes (Barnett and Binder, 1973; DeVay, 1956). The symptoms produced on the infection of mature mushroom sporophores by V.fungicola may be explained by the action of extracellular enzymes which degrade the mushroom cell wall, causing cell and ultimately tissue collapse. This constitutes a necrotrophic mode of nutrition, as defined by Cooke (1976) and Rudakov (1978).

A similar pattern of parasitism has been described for other Hyphomycete-Basidiomycete interactions. With Hypomyces species attacking Boletus edulis Fr ex Bull. (Touze-Soulet et al, 1980) extracellular enzymes of both H.chrysospermus Tul. and H.chlorinus Tul. caused degradation of the host cell wall and the greater degree of necrotrophy exhibited by H.chrysospermus was linked to higher chitinase activity by this pathogen. The parasitism of A.bisporus by Mycogone perniciosa also appears to be primarily enzymatic and is similar in mechanism to parasitism by V.fungicola (Vincent-Davies, 1973). The symptoms produced on infection of sporophores with M.perniciosa are however generally more

extensive than those produced by V.fungicola but the factors which limit lesion size in V.fungicola infections are not understood.

(ii) The production of deformities of mushroom tissue by V.fungicola.

The characteristic sporophore deformities produced after infection of the initials by V.fungicola suggest that the pathogen interferes with the normal processes of growth and development. The mechanics of growth of healthy sporophores has received limited attention (Bonner et al, 1956; Craig et al, 1977; Schmitz, 1842) but the control of development is poorly understood (Gruen, 1963).

The rapid growth of the sporophore is due mainly to the expansion of cells differentiated early in development (Bonner et al, 1956) and it is with such rapidly expanding cells that V.fungicola is likely to make contact in casing. Schmitz (1842) demonstrated that the elevation of the sporophore above the casing surface is due largely to the expansion of cells in the uppermost region of the stipe. This expansion also occurs in cut sporophores and, to a lesser extent, in excised stipes (Gruen, 1963). The rapidly expanding stipe apex tissue of cut sporophores was used as a model system to investigate the mechanism of deformation of mushroom tissue by V.fungicola.

Materials and Methods

Inverted sporophores at growth stages 3 - 5 were inoculated with 250 µl of conidial suspension (1×10^6 ml^{-1}) of V.fungicola isolates G3, G4 and C1 around the stipe apex and were incubated at room temperature in humid chambers. Controls were similarly treated with sterile distilled water. After 3 days the stipes were examined for deformities.

Pieces of tissue (c. 4 x 4 x 2 mm) were cut from comparable regions of the outer layers of apices of both healthy and distorted

stipes from inoculated sporophores and were macerated in 6% KOH for 17 h on a wrist action shaker (Craig, 1979). Groups of separated cells were photographed at random and cell lengths and breadths were measured using an opisometer. These measurements were compared for the healthy and distorted tissue using Student's t test. Tissue from naturally infected distorted tissue was similarly treated and the cell morphology was compared with healthy controls.

Pieces of tissue, c. 2 mm^3, were cut from the outer layers of unexpanded stipe apices of sporophores at growth stage 3 and were incubated in a cell free V.fungicola culture filtrate (prepared as described on page 50) for 4 days at 20°. The tissue blocks were then macerated and the cell dimensions measured and compared with those of cells incubated in uninoculated culture medium.

Cell free culture filtrate and an extract of sclerodermoid sporophores prepared in a similar way to the tissue extracts described on page 50 were tested for the ability to cause distortion of expanding stipe tissue. Inverted sporophores at growth stage 3 - 4 were either treated with 250 µl of filtrate or extract pipetted around the stipe apex or stipe apices were injected with 50 µl of filtrate or extract. Control sporophores were treated with either culture medium alone or with an extract of healthy sporophores.

To determine the qualitative effect of inoculation with V.fungicola on stipe expansion, the lengths and widths of stipes of cut sporophores (growth stages 3 and 4) were measured before inoculation with 250 µl of a V.fungicola conidial suspension (c. 2 x 10^5 ml^{-1}) pipetted around the stipe apex or with 150 µl of the same suspension injected at three points in the expansion region and again after 3 days incubation in humid chambers at room temperature. Stipe expansion was compared to controls treated with sterile distilled water. To account for

differences in the initial lengths and widths of the stipes, expansion was standardised by dividing the change in dimension by the original dimension. The experiment was repeated three times.

The effect of inoculation of excised stipes with V.fungicola was also examined. Uniform unexpanded stipes (growth stage 3) c. 20 mm long were placed, base down, on moist filter paper and were inoculated with 0.5 ml of a V.fungicola conidial suspension. Control stipes were treated with sterile distilled water. The stipes were incubated in humid chambers for 3 days at room temperature and the morphology of inoculated and control stipes was compared.

Further experiments investigated the qualitative effect of inoculation with V.fungicola on different regions of excised stipes. Uniform, unexpanded stipes were cut into three sections, top, middle and bottom, and the lengths of each section (between 5 and 10 mm) were measured. The sections were placed base down on moist filter paper and 250 µl of a V.fungicola conidial suspension were pipetted over them. Controls were treated with sterile distilled water. The sections were incubated in humid chambers for 3 days at room temperature and were measured again. Expansion of the sections was expressed as change in length divided by the original length.

Results

After incubation for 3 days, during which time the stipes had expanded, the apices of stipes of some sporophores inoculated with all three V.fungicola isolates had become deformed. In each trial consisting of 6 inoculated and 6 control sporophores, the percentage of deformed stipes varied from 0 - 80%.

The morphology of the deformities was variable, but commonly consisted of localised swelling of the tissue in a ring round the stipe (Plate 42). When the drop of inoculum rested to one side of the stipe,

Plate 42 Localised swelling of stipe apex tissue 3 days after inoculation of the apex with a conidial suspension of V.fungicola G3. The control on the right hand side was treated with sterile distilled water. The sporophores were at growth stage 3 when treated.

the deformity was localised in that region. Longitudinal sections of stipes revealed that the deformities were frequently hollow, the outermost tissue having folded out to leave a cavity beneath.

A fragment of tissue excised from the outer layer of a deformed stipe and a comparable fragment from an uninoculated control are shown in Plates 43 and 44. In the control tissue the hyphae tended to be arranged in parallel files, whilst in the deformed tissue the individual hyphae were contorted and their linear organisation was less marked. Contortion of the hyphae was more apparent when the tissue was macerated to separate the cells (Plates 45 and 46). Cells in macerates of naturally infected but similarly deformed tissue were similarly contorted.

Cells from artificially inoculated deformed tissue were significantly ($P = 0.05$) longer and thinner than those from healthy tissue (Table 10).

Table 10. Dimensions of cells in macerates of deformed, inoculated stipe apex tissue and in water treated controls.

	Mean cell dimension (μm ± SE)	
	Length	Breadth
Inoculated	113.0 ± 3.8[a] (112)	10.7 ± 0.4[b] (112)
Water control	89.9 ± 8.7[a] (100)	16.7 ± 0.7[b] (100)

a mean lengths significantly different at $P = 0.05$

b mean breadths significantly different at $P = 0.05$

Figures in parenthesis indicate the number of cells measured.

When unexpanded stipe tissue was incubated in V.fungicola culture filtrate, there was a significant ($P = 0.05$) decrease in both the width

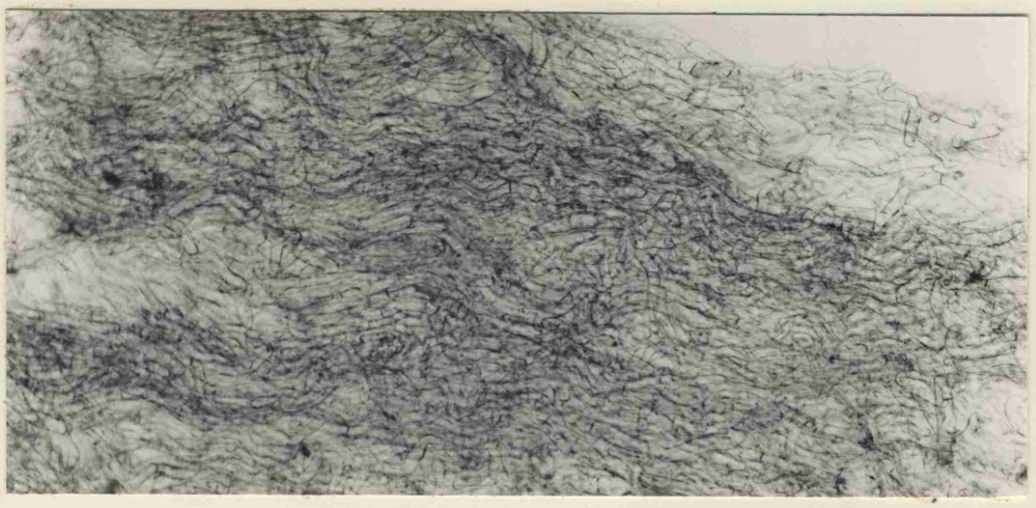


Plate 43 A fragment of tissue excised from an artificially inoculated and distorted stipe apex. Note the contortion of individual cells and the lack of linear organisation of the hyphae within the tissue. x 120

Plate 44 A fragment of tissue excised from a water treated undistorted stipe apex. The individual cells are less contorted than those in the inoculated tissue and tend to be arranged in parallel files. x 120

Plate 45 Cells from a macerate of inoculated and distorted stipe apex tissue. Note the contortion of the cells and the poor continuity of the cell walls (arrowed). x 800

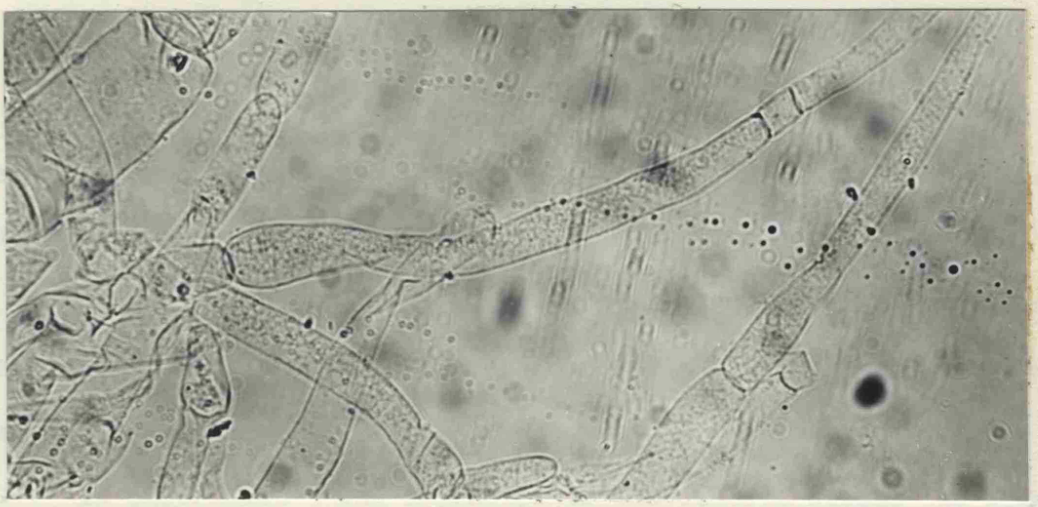


Plate 46 Cells from a macerate of water treated stipe apex tissue. The cells are generally straight and the cell walls intact. x 800

and length of cells compared with control tissue incubated in uninoculated culture medium (Table 11).

Table 11. Dimensions of cells in macerates of tissue incubated in V.fungicola culture filtrate or uninoculated culture medium for 4 days at 20°.

	Mean cell dimension µm ± SE	
	Length	Breadth
Culture filtrate	65.0 ± 2.8 (78)[a]	12.7 ± 0.4 (145)[b]
Uninoculated culture medium	71.1 ± 2.2 (65)[a]	17.3 ± 0.6 (119)[b]

a mean lengths significantly different at $P = 0.05$

b mean breadths significantly different at $P = 0.05$

Figures in parentheses indicate the number of cells measured.

Treatment of expanding, attached stipes with either culture filtrate or an extract of sclerodermoid sporophores did not induce deformities in the zone of expansion.

The effect of inoculation with a V.fungicola conidial suspension on the lengths and diameters of expanding, attached stipes is shown in Table 12.

In experiment 1 the expansion of the control stipes was significantly ($P = 0.05$) greater than that of the inoculated ones although the overall expansion was less than in subsequent experiments, probably because of the greater physiological age of the sporophores. Although the expansion of control stipes was marginally greater than that of the inoculated stipes in experiments 2 and 3, the differences were not

Table 12. Mean standardised change in dimension after 3 days' incubation at room temperature of attached stipes inoculated with either a V.fungicola conidial suspension or with sterile distilled water. Experiments 1 and 2 were inoculated by pipetting 250 µl of conidial suspension around the stipe apices and experiment 3 by injection of 50 µl of conidial suspension at 3 equidistant points around the stipe apices.

Experiment		Sporophore growth stage	Number of distorted sporophores	Mean standardised change *	
				Inoculated	Control
1.	Length	4	3	+0.27[a] ± SE 0.05	+0.42[a] ± SE 0.02
	Width	ND	ND	ND	ND
2.	Length	3	0	+0.79 ± 0.07	+0.80 ± 0.20
	Width	3	0	-0.12[b] ± 0.02	-0.04[b] ± 0.02
3.	Length	3	0	+0.75 ± 0.08	+0.89 ± 0.07
	Width	3	0	-0.02 ± 0.02	-0.07 ± 0.02

* standardised change = $\frac{\text{dimension after treatment}}{\text{original dimension}}$ mm

ND not determined

a Significant difference in mean standardised change in length ($P=0.05$)
b Significant difference in mean standardised change in width ($P=0.05$)

significant (at $P = 0.05$). The diameters of both inoculated and control stipes in these two experiments had decreased to a significantly ($P = 0.05$) greater extent than the control because of the collapse of tissue associated with lesion development. In experiment 3, where the different method of inoculation did not result in such marked tissue collapse, the differences between inoculated and control stipe diameters were not significant at $P = 0.05$. Tissue deformity i.e. localised swelling of the stipe, was only produced in experiment 1.

The results of the experiments on the expansion of excised stipes were also variable. Deformities were not produced on every inoculated stipe and when they occurred they were generally less severe than those on intact sporophores (Plate 47). As reported by Craig et al (1977) there was a gradient of expansion down the stipe with the apical sections expanding the most whilst expansion in the basal sections was variable (Fig. 16). There were no significant differences ($P = 0.05$) in expansion between inoculated and control sections, irrespective of the region from which they were taken, but it is interesting to note that inoculation tended to increase the expansion of the basal sections relative to the controls.

Discussion

When V.fungicola invades rapidly expanding stipe apices the tissue may become deformed and the expression of the deformities suggests an interference with the control of stipe expansion. The production of deformities is variable and the factors that determine the occurrence and magnitude of deformation are not clear.

One possible explanation for this variability is that, for deformation to occur, infection by V.fungicola must coincide with a particular physiological state of the stipe. Generally, the most frequent

Plate 47

Top row: control stipes treated with sterile distilled water.

Bottom row: deformities (arrowed) produced by the inoculation of stipes excised from sporophores at growth stage 3 with a V.fungicola conidial suspension.

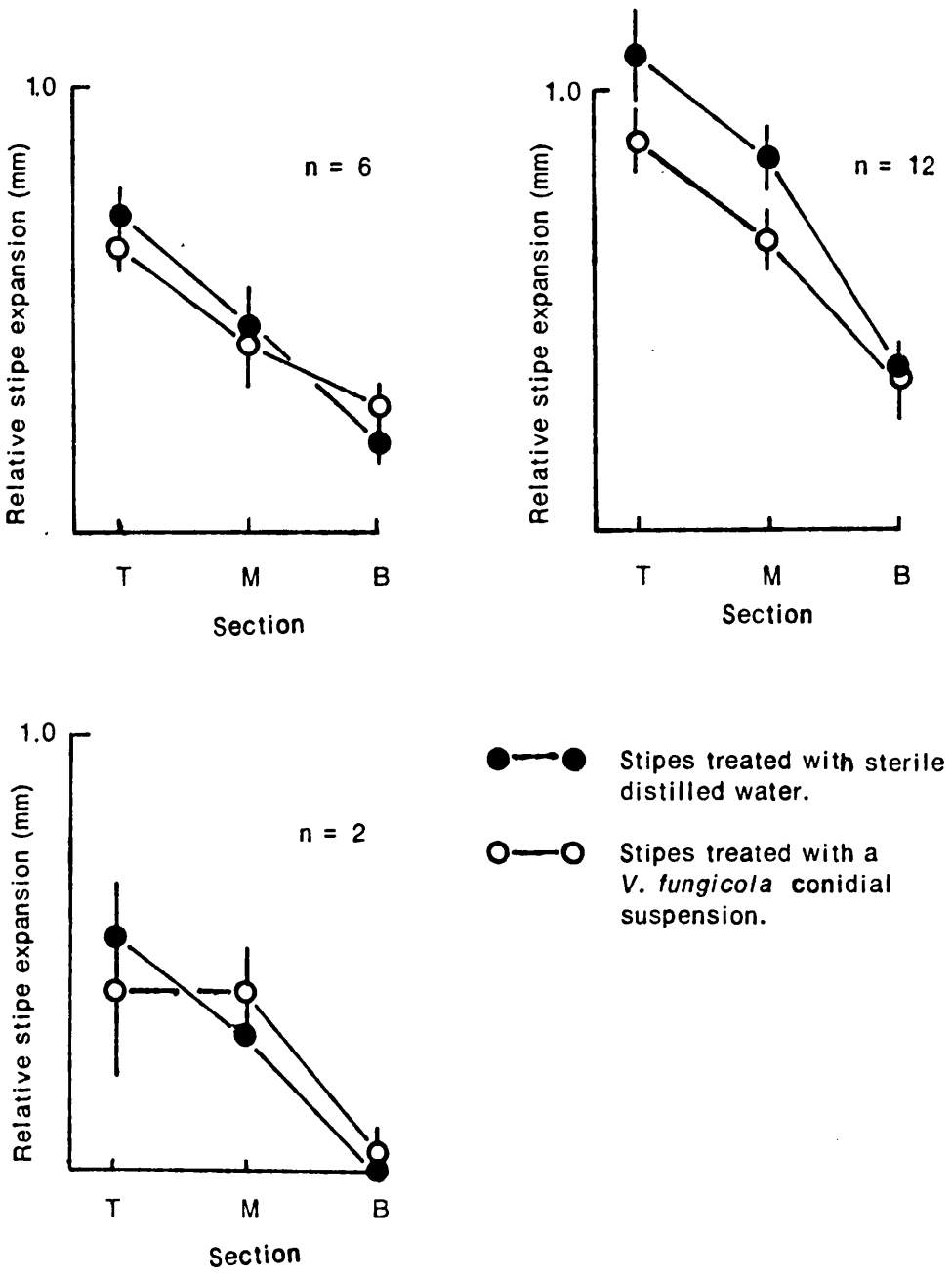


Fig.16. The relative expansions of different sections (T, top; M, middle; B, bottom) of excised stipes either inoculated with *Vf.* or treated with distilled water. The results are of three separate experiments. n = the number of stipes used. Bars represent the standard error of the mean.

and severe distortions were produced when sporophores were inoculated at growth stage 3, prior to veil break, when the elongation rate of the stipe reaches its maximum (Gruen, 1963) which suggests that the rate of expansion of cells at infection may in part determine the degree of damage caused.

Gruen also demonstrated that stipe expansion was promoted in the presence of the gill lamellae and was retarded when stipes were decapitated. This slower rate of expansion may explain why deformities produced on excised stipes were less severe than those on intact stipes.

Changes in the dimensions of expanding cells induced by infection with V.fungicola may be responsible for the deformation of stipe apex tissue. Cells from distorted tissue of both artificially and naturally infected stipes appeared contorted and, in the former case, were significantly longer and thinner than cells from comparable uninoculated tissue. This suggests that V.fungicola either directly stimulates cell expansion or interferes with the normal regulation of expansion in rapidly expanding tissue.

For rapid cell expansion to take place, the mushroom cell walls must become less rigid and this is probably brought about by a change in the conformation of the wall polymers (Vincent-Davies, 1973). Since V.fungicola is capable of degrading the structural polymers of the mushroom cell wall, a change in the configuration of the polymers may render them more susceptible to enzymic attack and thus further promote wall plasticity and extension. The integrity of the walls of cells isolated from distorted tissue appears to be diminished compared to healthy controls (Plates 28 & 29).

Individual cells within inoculated, expanding tissue are likely to become deformed because of the forces preventing their linear expansion (eg. the pressure of surrounding tissue or the weight of the stipe or pileus) and the net effect of the contortions of individual cells is

to cause deformation of the tissue. This may explain why the increase in length of individual cells is not reflected in the increased expansion of the whole organ since, when distortions were produced as in experiment 1, the tissue buckled out rather than expanding in length and resulted in a smaller overall expansion of the stipe than in the controls.

In the experiments with excised stipe sections there was a consistent, although statistically insignificant, stimulation of expansion of inoculated basal sections compared to the controls. This is surprising in view of the small amount of expansion that occurs in the lower region of the stipe and the reasons for it in this case are obscure.

The stimulation of cell expansion could not be induced by V.fungicola culture filtrate and neither culture filtrate nor an extract of diseased sporophores reproduced the distortions on intact sporophores. This suggests that a close physical association between the host and pathogen is necessary for deformation to occur although it is possible that a labile extracellular factor or factors are involved. A third possibility is that expansion was retarded under the conditions of the assay of the effect of culture filtrate on cell expansion so that the distortion factor was not in contact with rapidly expanding cells. A comparison of the mean cell lengths from this experiment (65 and 71μm for culture filtrate and control respectively) with those of the tissue macerates (113 and 89 μm for inoculated and uninoculated tissue respectively) tends to support this.

Cells in the culture filtrate however expanded to a lesser extent than those in uninoculated culture medium. This suggests that, under the conditions of the assay, either a factor present in the culture filtrate inhibited cell expansion or that a factor present in the uninoculated

medium stimulated cell expansion. The possible nature of such factors is not known.

The consequences of infection of rapidly expanding and non-expanding mushroom tissue by V.fungicola are quite distinct. In the former, the primary effect is the promotion of cell expansion whilst in the latter it is cell collapse. Both however may be explained in terms of the weakening action of enzymes on the mushroom cell wall, the different symptoms produced reflecting the different physical states of the wall at infection. The development of sporophores consists of two distinct phases. Until the primordia reach approximately 2 cm in length growth is slow and is due mainly to cell divisions that differentiate the tissues of the mature sporophore. After a brief pause, the sporophore grows rapidly in all directions due mainly to the expansion of already differentiated tissue (Bonner et al, 1956; Hagimoto, 1964) although Craig (1979) has reported that some cell division occurs in the second growth phase.

Young primordia are susceptible to infection by V.fungicola and early colonisation if initials may result in cessation of growth (W.C.Wong; pers. comm.). When such primordia are infected, the small mass of host tissue and consequently large pathogen/host ratio is likely to lead to more serious effects on development than infection at later stages of differentiation and could completely arrest sporophore growth.

As the primordium starts its rapid expansion, infection may lead to an interaction between the pathogen and host tissue similar to that demonstrated for rapidly expanding stipe apex tissue and may consequently result in tissue deformation. If infection occurs in the early stages of sporophore expansion when the pathogen/host ratio is still large there may be a serious loss of control of the differentiation of the

sporophore tissues resulting in completely undifferentiated ('sclero-dermoid') sporophores. As the sporophore continues to grow, the overall rate of expansion decreases and expansion tends to become localised in the stipe and because of its greater size, infection is also likely to become more localised. Interference with the control of growth at this stage of development may result in less severe distortions or locally expressed symptoms such as 'bubbles' on the pileus or peeling of stipe tissue. The latter symptom is often associated with a lesion to one side of the stipe and a curvature of the stipe away from the lesion. Localised expansion of the stipe in the region of the lesion could explain the stipe curvature and if the forces produced by expansion exceed the physical strength of the tissue, tearing of the tissue (stipe peeling) is the likely outcome.

CHAPTER 5

STUDIES ON THE INTERACTION BETWEEN V.FUNGICOLA AND THE SPOROPHORE SURFACE MICROFLORA

The presence of microorganisms on aerial plant parts has been recognised since the turn of the century (see Leben, 1961, for references) and Potter (1909) suggested that the indigenous microflora may influence the course of development of plant disease. However, little work was done on the influence of surface microorganisms on disease until attention was again drawn to aerial plant parts as 'an ecologically neglected milieu' (Ruinen, 1961). Several reports have since shown that microorganisms, particularly bacteria, can influence the infection of both fungal and bacterial pathogens (Crosse, 1971; Blakeman and Brodie, 1976; Fraser, 1971; Leben & Daft, 1965).

Both light and scanning electron microscope studies of the infection on mushroom sporophores by V.fungicola revealed large numbers of bacteria within infection drops. Experiments were performed to determine whether the indigenous cap microflora could influence pathogenesis.

Materials and Methods

1. Isolation of microorganisms.

Fungi and bacteria were isolated from sporophore surfaces by rolling freshly picked sporophores at growth stage 4 over the surface of 2% malt or nutrient agar in Petri dishes and incubating at 22° and 25° for fungi and bacteria respectively. The bacteria were tentatively identified to genus level using the scheme of Bradbury (1970).

Bacteria were also isolated from V.fungicola infection drops. Drops (c. 50 µl) of V.fungicola conidial suspension in sterile distilled

water (c. 1 x 10^6 ml^{-1}) were placed on sporophore surfaces and, after 42 h incubation at room temperature, were recovered using sterile syringes. The suspension was streaked over nutrient agar plates which were than incubated at 25°. Three morphologically distinct isolates were obtained (M1, M2 and M3) and were maintained in pure culture.

2. Interaction between isolates from sporophores and V.fungicola in vitro.

Discs of colonies of potential fungal antagonists, 6 mm in diameter, were placed 4 cm from the margin of a 7 day old V.fungicola colony on malt agar. The cultures were incubated at room temperature and were periodically examined for evidence of antagonism between the colonies.

Suspensions of the bacteria isolated from healthy sporophore surfaces were streaked at right angles to a streak of V.fungicola conidia on malt agar. The bacteria isolated from V.fungicola infection drops were treated similarly but the media used were malt agar, PDA, 17%, prune agar and Czapek-Dox agar. The cultures were incubated at 22° and were periodically examined.

Washed suspensions of V.fungicola conidia from a 7 day old PDA culture and bacterial cells from 2 day old nutrient agar cultures of the 3 infection drop isolates were prepared to give final concentrations of 1 x 10^6 and 5 x 10^7 propagules ml^{-1} respectively. The suspensions were pipetted on to cavity slides both with and without liquid Czapek-Dox medium as a nutrient source. Five replicate slides were prepared of each treatment (Table 13) and the treatments were repeated for all 3 bacterial isolates.

Table 13. Composition by volume (µl) of each treatment.

Treatment	Conidial suspension	Bacterial suspension	Sterile water	Liquid Czapek-Dox
1	50	20	0	10
2	50	0	20	10
3	50	20	10	0
4	50	0	30	0

The percentage germination of conidia in different treatments was compared, using Student's t test.

To determine whether germination of V.fungicola conidia was affected by the presence of living bacterial cells or by extracellular products of either bacterial or V.fungicola growth, washed suspensions of conidia and of two bacterial isolates (M2 and M3) were obtained as previously described. Sterile boiling tubes were prepared, containing (a) 2 ml each of conidial and bacterial suspensions, (b) 2 ml each of bacterial suspension and sterile water, (c) 2 ml each of conidial suspension and sterile water. The boiling tubes were placed in a shaking incubator at 22° and after 24 h the suspensions were sterilised by membrane filtration. Drops of the sterile leachates (25 µl) were pipetted onto cavity slides with an equal volume of freshly prepared washed V.fungicola conidial suspension and 10 µl liquid Czapek-Dox medium.

The slides were incubated at 20°C and the percentage germination from 3 replicates was measured after 24 h.

3. The effect of conidial suspensions on the indigenous bacterial populations of sporophore surfaces.

Washed conidial suspensions (c. 1 x 10^6 ml^{-1}) of V.fungicola and,

for comparative purposes, of the non-pathogenic species Verticillium lateritium (Rabenh) were prepared and 50 µl drops of the suspensions and sterile water were pipetted on to the surfaces of freshly picked, healthy sporophore at growth stage 4. After 0, 3, 12, 24 and 42 h incubation in moist chambers at room temperature, 10 drops of each treatment were removed with a sterile syringe and 50 µl from each sample subjected to 1000 and 10,000-fold dilution in sterile distilled water. Nutrient agar plates were inoculated by spreading 50 µl of each dilution over the surface of three replicate plates with a flamed, bent glass rod and incubated at 22° for 4 days, after which the bacterial colonies were counted.

To check for bacterial contamination of the original conidial suspensions, 50 µl of each were also spread onto nutrient agar plates. No bacterial colonies developed after 4 days.

After a further 7 days incubation, the numbers of colonies of V.fungicola and V.lateritium that developed on the plates were also counted.

Eight morphologically distinct bacterial isolates were obtained in pure culture from the dilution plates (3 from the water control, 3 from V.lateritium and 2 from V.fungicola) and were tested for pathogenicity towards mushrooms using the method of Gandy (1968). Blocks of tissue (c. 1 x 0.5 x 0.5 cm) were cut from sporophore caps and the surface layers were aseptically removed. The blocks were transferred to sterile slides in Petri dishes lined with moist filter paper and were inoculated with a turbid suspension of the bacterial isolates. Control tissue blocks were treated with sterile distilled water. The tissue blocks were incubated at 22°C and periodically examined for symptoms of browning and collapse.

The 8 bacterial isolates were also tested for antagonism towards V.fungicola and V.lateritium on PDA as described above for the isolates

obtained from healthy sporophore surfaces.

The leakage of nutrients from sporophore surfaces into water drops was estimated by measuring the increase in reducing sugars in the drops with time. Drops of sterile distilled water (50µl) were pipetted onto the surfaces of sporophores which were incubated at room temperature in humid chambers. After 0, 5, 14, 20 and 24 hours c. 1 ml of exudate was removed using a clean syringe and the reducing sugar content (expressed as glucose equivalents) was determined by the colorimetric methods of Nelson (1944) and Somogyi(1945).

Axenic sporophores of A.bisporus strain Darlington 621 produced by the method of Long and Jacobs (1974) were inoculated with a V.fungicola conidial suspension under sterile conditions. The symptoms produced were compared with those characteristic of infections of non-sterile sporophores.

Results

Twenty-six fungal and 15 bacterial isolates were obtained from healthy sporophore surfaces. The most frequently isolated fungal genera were Penicillium, Trichoderma, Pythium and Cladosporium. Pseudomonas was the most frequently isolated bacterial genus, whilst yellow isolates keyed to both Erwinia and Flavobacterium.

Only one of the fungal isolates (Penicillium sp.) inhibited the growth of V.fungicola in the absence of colony contact although another Penicillium isolate completely overgrew the V.fungicola colony. Several of the Cladosporium and Pythium isolates were overgrown by V.fungicola and frequent associations between hyphae of V.fungicola and these test fungi similar to those observed with A.bisporus were noted.

A Pseudomonas sp. isolated from healthy sporophores inhibited the germination and growth of V.fungicola on agar. Within 1 cm of the

bacterial colony hyphae and conidiophores were flexuous and sporulation was poor.

Of the 3 bacterial isolates from V.fungicola infection drops tested for antagonism on agar, one (M3) also inhibited germination, growth and sporulation of V.fungicola although this was only pronounced on PDA.

The effect of the 3 bacterial isolates from infection drops on the germination of conidia in suspension is shown in Table 14. In the presence of exogenous nutrients, isolate M2 significantly ($P = 0.05$) decreased the percentage germination of conidia whilst the other two isolates had no effect. Without exogenous nutrients, the overall percentage germination was reduced and was further decreased by both isolates M1 and M2. Under these conditions however, isolate M3 significantly stimulated germination.

The cell free leachates of isolates M2 and M3 had no effect on conidial germination compared to the controls (Table 15) which suggests that living bacterial cells are necessary to influence germination.

The changes in numbers of bacteria in drops of conidial suspensions of V.fungicola, V.lateritium and water on sporophore surfaces are shown in Figure 17. The results were variable with the standard error ranging from 2 to 67% of the mean, but there were clear differences between the pathogenic and non-pathogenic Verticillium species.

Starting from approximately equal populations, bacteria multiplied rapidly in all drops, but at different rates. After 3 hours, the number of bacteria in V.fungicola infection drops was up to two orders of magnitude greater than in either the V.lateritium suspension or in sterile distilled water. The multiplication rate decreased after 3 hours and, in the V.lateritium suspension and sterile distilled water, remained fairly steady for the following 39 hours. Allowing for the

Table 14. Mean percentage germination of V.fungicola conidia after 24 h at 20°C in the presence of bacteria isolated from infection drops.

	TREATMENTS							
	1. Nutrients + bacteria		2. Nutrients - bacteria		3. No nutrients + bacteria		4. No nutrients - bacteria	
Isolate		SE		SE		SE		SE
M1	95.9	0.5	95.5	0.5	3.8	1.4[b]	14.6	3.5[b]
M2	70.8	4.2[a]	91.2	2.8[a]	4.8	1.1[c]	15.7	2.0[c]
M3	95.7	0.9	95.7	0.8	94.6	0.9[d]	25.1	1.6[d]

a, b, c, d. Pairs with the same letter are significantly different at $P = 0.05$.

Table 15. Mean percentage germination of V.fungicola conidia after 24 h at 20°C in the presence of leachates from infection drops.

	LEACHATE							
	Bacteria and conidia		Bacteria alone		Conidia alone		Water control	
Bacterial isolate		SE		SE		SE		SE
M2	83.8	4.1	87.1	1.1	82.0	6.5	86.5	1.2
M3	96.0	0.9	95.7	0.8	96.2	0.9	95.4	0.5

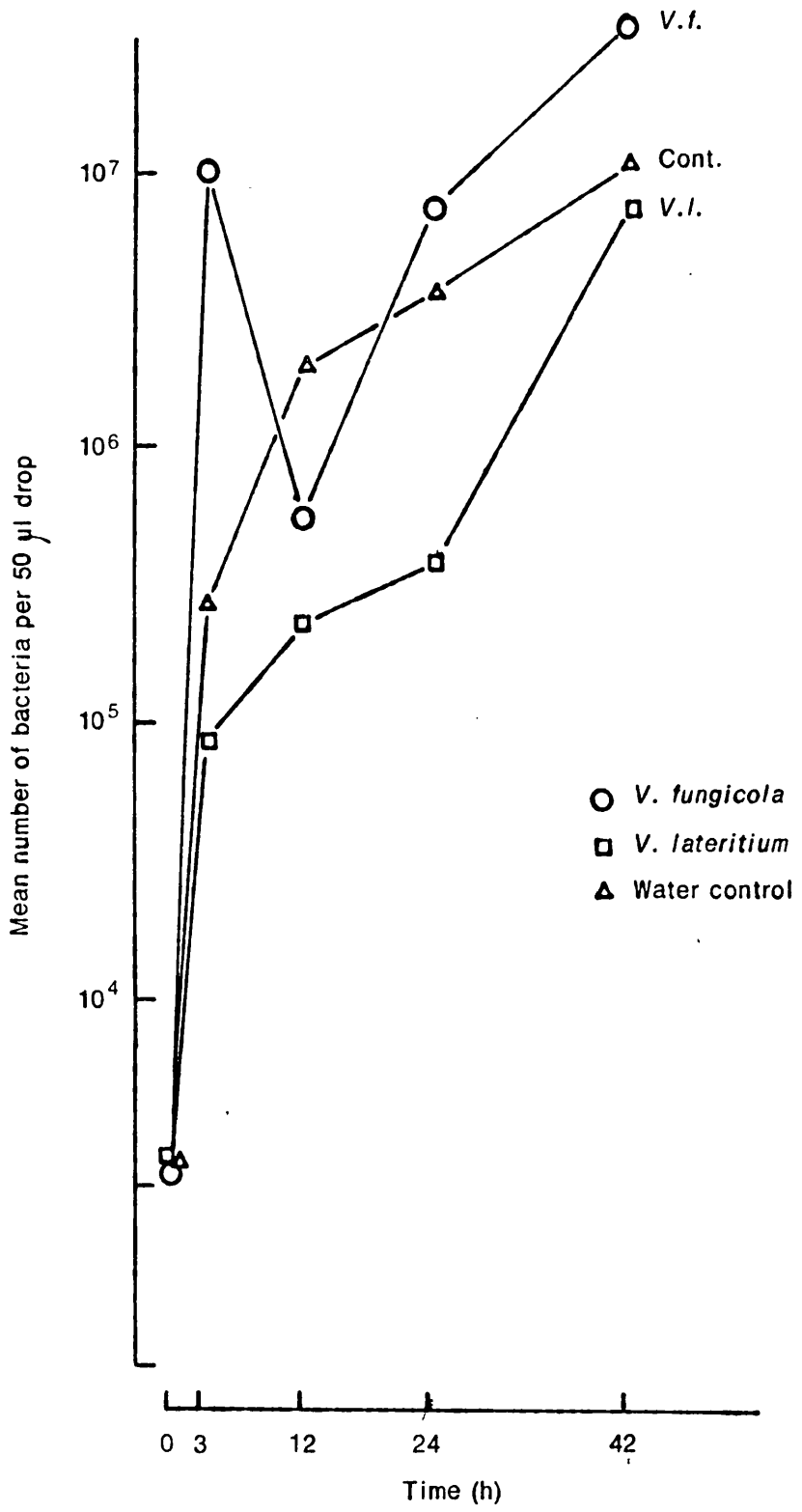


Fig.17. Changes in bacterial numbers in conidial suspensions of *V. fungicola*, *V. lateritium* and drops of sterile distilled water incubated on mushroom caps with time. Colony counts were made after 4 d incubation at 22°C. Each point represents the mean of 3 replicates.

large variation between replicates, the V.lateritium suspension contained similar numbers of bacteria to the water control. However, the numbers of bacteria in the V.fungicola infection drops decreased rapidly between 3 and 12 hours then increased equally rapidly so that, after 24 h, the infection drops again contained the greatest number of bacteria.

There were also differences in the types of bacteria present in the different drops. After 24 hours, the V.lateritium suspension and sterile distilled water drops contained 7 morphologically distinct bacteria, whilst in the V.fungicola drops, one colony type, isolate F24, predominated (Plate 48).

Two of the 8 isolates, one from the water drops and the other, F24, caused slight browning of mushroom tissue blocks after 48 h but the symptoms were not comparable to those caused by Pseudomonas tolaasi (Paine) infections.

None of the bacterial isolates obtained in this experiment affected the germination and growth of V.fungicola on PDA, but the growth of V.lateritium was inhibited by two isolates, one a yellow bacterium from the V.lateritium suspension drops and the other, isolate F24 (Plate 49).

The number of V.lateritium colonies recovered from the suspension drops decreased rapidly over the first 3 hours but then levelled out to approximately 4% of the original inoculum. By contrast, the numbers of V.fungicola colonies recovered decreased steadily up to 12 h after inoculation, when the inoculum recovered was equivalent to 2% of the original. After this time, no further colonies developed (Fig. 18).

The amounts of reducing sugars leached into water drops on sporophore surfaces are shown in Figure 19. There was an initial rapid leakage corresponding to the period of rapid bacterial multiplication, followed by a decline. However, after 20 h, leaching again

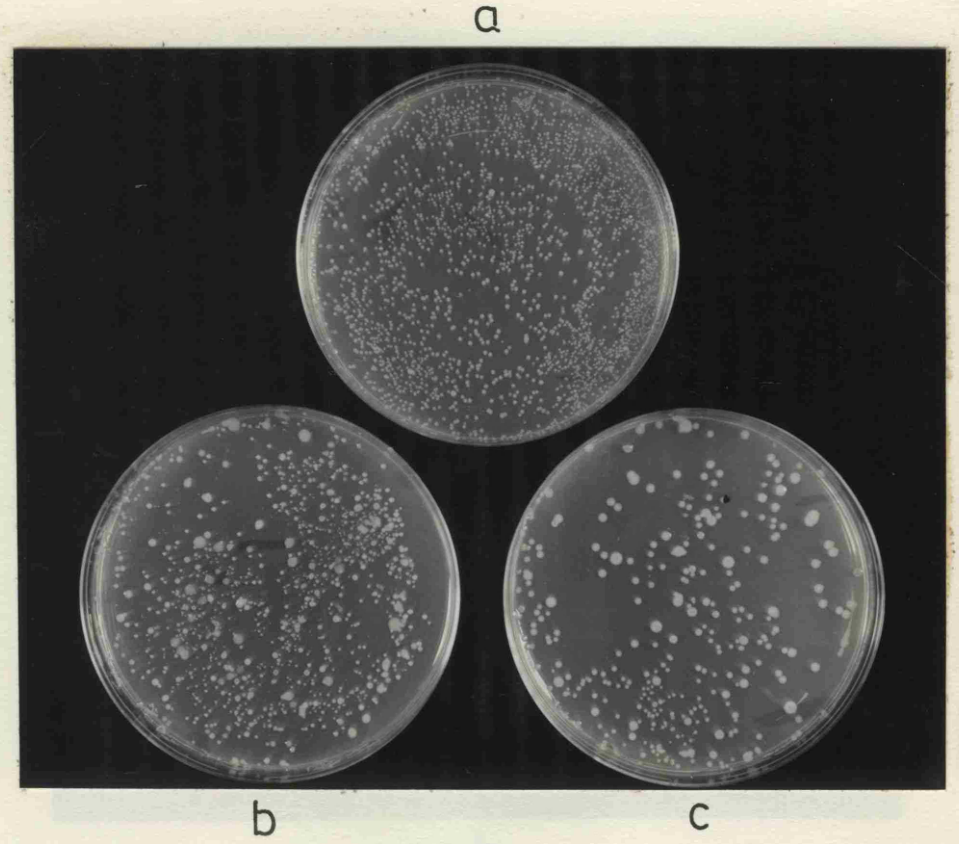


Plate 48 Nutrient agar dilution plates showing the qualitative and quantitative differences in the bacterial colonies recovered from V.fungicola and V.lateritium conidial suspensions and sterile distilled water after 24 h incubation on sporophore surfaces.

a. V.fungicola

b. V.lateritium

c. Water control

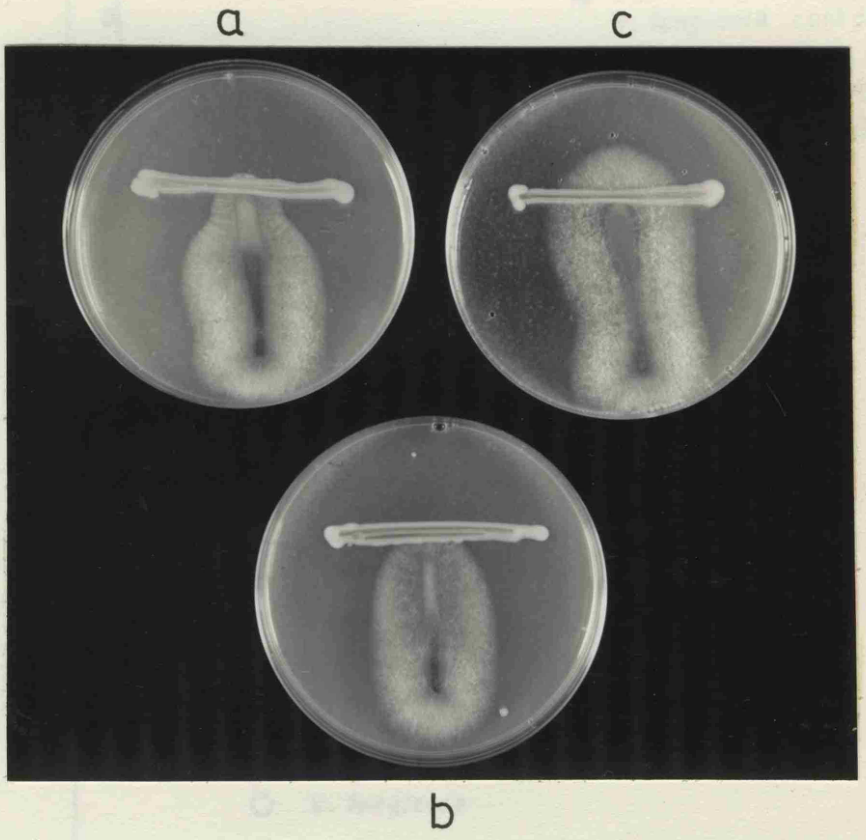


Plate 49 Antagonism of bacterial isolates F24 and L24 obtained from sporophore surfaces towards V.lateritium after 5 d incubation at 22°C on PDA.

a. Isolate L24

b. Isolate F24

c. The non-antagonistic isolate C24

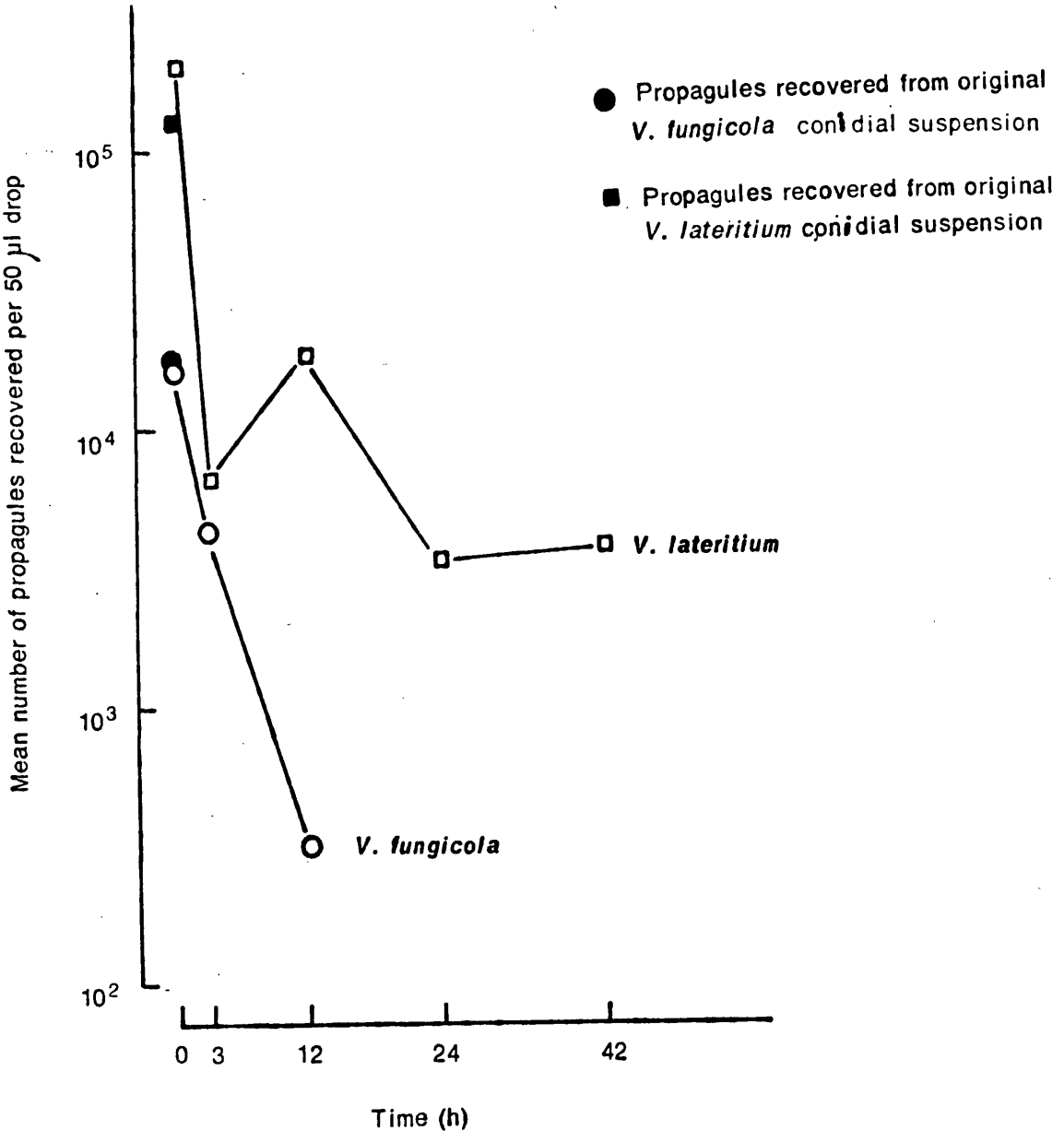


Fig.18. Changes in the numbers of *V. fungicola* and *V.lateritium* colonies recovered from drops of conidial suspensions incubated on mushroom caps with time. Colony counts were made after 11 d incubation at 22°C. Each point represents the mean of 3 replicates

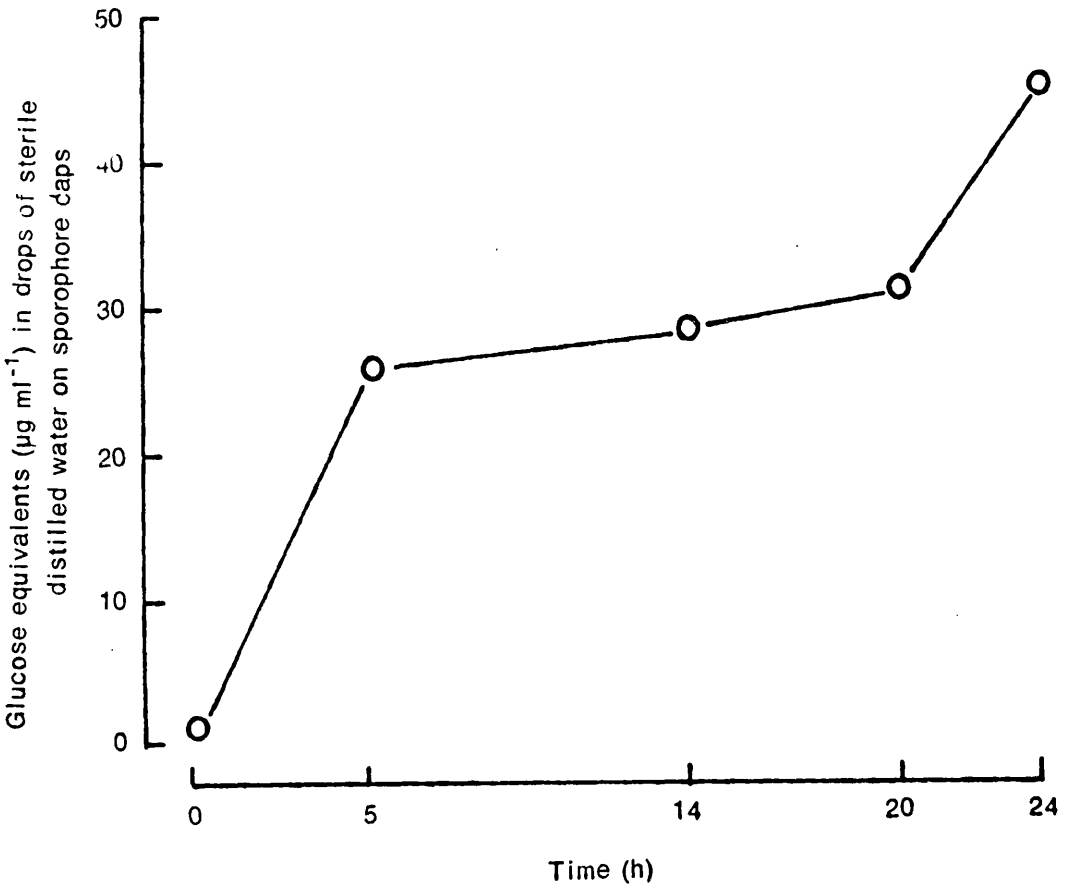


Fig.19. Nutrient exuded from sporophore caps (expressed as glucose equivalents, µg ml^{-1}) into drops of sterile distilled water with time.

returned to the initial rate.

V.fungicola caused both distortions and cap lesions on axenically grown sporophores, but in the absence of bacteria, the rapid browning normally associated with the early stages of infection was reduced.

Discussion

Although both fungi and bacteria isolated from healthy sporophore surfaces were capable of antagonising the germination and growth of V.fungicola in dual cultures, it would be unwise to extrapolate from the results obtained *in vitro* to the situation on the sporophore surface. The different response of V.fungicola to isolate M3 in two different assays (agar plate and conidial germination in suspension) serves to underline the influence of test conditions on the development of antagonism.

The isolation technique used to investigate the sporophore microflora may have been selective for fast growing fungal genera and consequently slower growing members of the microflora may have been overlooked.

The response of V.fungicola conidia to the three bacterial isolates from infection drops differed both between and within isolates depending on the presence or absence of exogenous nutrients. Similarly varied results were obtained by Clark and Lorbeer (1976) and Fraser(1971) in studies of the effect of bacterial isolates from leaves on the germination of Botrytis cinerea Pers. ex Fr. conidia. In the former study two isolates also stimulated the germination of conidia in the absence of exogenous nutrients, as did isolate M3 from V.fungicola infection drops.

The effects of bacteria on fungal spore germination *in vitro* may be brought about by several different mechanisms which include

antibiotic production, changing growth conditions (eg. pH), lysis and nutrient competition (Blakeman & Brodie, 1976; Skidmore, 1976). The results of the bacterial cell leachate experiment, however, suggest that the presence of living bacterial cells is necessary to influence the germination of V.fungicola conidia and that the production of antibiotics or modification of growing conditions are unlikely to have effected the observed results. Whilst in some instances bacteria were found to be closely associated with conidia and germ tubes, there was no evidence of lysis of the latter. The decreased percentage germination of V.fungicola associated with isolates M1 and M2 in the absence of exogenous nutrients is most likely to be due to competition for nutrients, which may have originated from the conidia themselves (Blakeman & Brodie, 1976). The stimulation of germination by isolate M3 is likely to be due to autolysis and release of nutrients from the bacteria under conditions of nutrient deficiency. With isolates M1 and M3 the effects on germination were nullified when nutrients were not limiting, but depletion of nutrients by isolate M2 may have been sufficiently great to decrease germination even when exogenous nutrients had been supplied.

The leakage of nutrients from plant surfaces into water drops has been well documented (Godfrey, 1976; Tukey, 1971) and has been shown to influence the development of several pathogens (Blakeman, 1971; Brown, 1922). Exudates are also thought to influence the indigenous bacterial population of leaf surfaces (Fraser, 1971) and the initial increase in numbers of bacteria in water drops on sporophore surfaces corresponded to a rapid leakage of nutrients from the sporophore. The presence of conidia of V.fungicola in water drops resulted in the development of a very large but unstable population of bacteria within 3 hours. Although a direct stimulatory effect by the conidia on the

bacteria cannot be ruled out, it is more likely that the vast increase in bacterial numbers reflects an increased leakage of nutrients from the sporophore mediated by the pathogen. This suggests that the first effects of V.fungicola conidia on the sporophore surface are very rapid and probably occur prior to germ tube extrusion. An increase in the numbers of bacteria in infection drops has also been reported for Mycosphaerella liguicola Baker, Dimock and Davis conidia on Chrysanthemum leaves (Fraser, 1971).

The decrease in bacterial numbers in the infection drops between 3 and 12 h suggests that autolysis occurred, probably as a result of the nutrients which supported the initial rapid multiplication having become depleted.

Despite the large numbers of bacteria in infection drops, some of which may be inhibitory to V.fungicola, all the drops produced lesions bearing sporulating mycelium. This suggests that if nutrient competition does occur in infection drops, V.fungicola has a selective advantage over the bacteria. One possible explanation for this is that the V.fungicola hyphae penetrate below the surface layer at an early stage and thus escape the influence of the bacteria. The inability to recover V.fungicola from infection drops after 12 h may also reflect the penetration or adhesion of germ tubes to the sporophore. Alternatively, the predominance of one bacterial type (F24) in V.fungicola infection drops might suggest that the rapid leakage of nutrients from mushroom cells preferentially selects bacteria which, like isolates M1 and M3, have no effect on V.fungicola when nutrients are not a limiting factor. Interestingly, isolate F24 was not antagonistic towards V.fungicola on PDA but inhibited the growth of the non-pathogenic species V.lateritium.

The pattern of bacterial multiplication in drops of conidial suspensions of V.lateritium was similar to the water controls although

the bacterial numbers were slightly lower. Conidia of V.lateritium have a nutrient requirement for germination (Tsuneda & Skoropad, 1978) and, whilst they were not observed to germinate on sporophore surfaces, germination was enhanced by a membrane filtered sporophore exudate. It is therefore possible that with this fungus, competition for nutrients by cap bacteria prevents germination on the sporophore surface. The lack of germination and germ tube penetration may also explain why more conidia were recovered from the suspension drops after 42 h than with V.fungicola.

None of the bacterial isolates proved to be pathogenic to mushroom tissue. The production of symptoms by the brown blotch bacterium Ps. tolaasi is thought to be due to increasing numbers of the indigenous population of the pathogen in water films on the sporophore surface, the bacteria using exuded nutrients for growth. The occurrence of the bacterial pathogen in a V.fungicola infection drop in which the nutrient content is increased is likely to lead to a rapid increase in pathogen numbers and lesion formation. In this context, it is interesting to note that the initial browning symptoms of sporophore infection by V.fungicola and Ps.tolaasi are often very similar. The reduced browning on infection of axenic sporophores with V.fungicola and the frequently observed browning of tissue below water drops suggests that, even in the absence of Ps.tolaasi, the initial symptoms of infection associated with V.fungicola may in part be due to bacteria.

CHAPTER 6

TAXONOMY AND ECOLOGY OF VERTICILLIUM SPECIES ISOLATED FROM NATURAL SUBSTRATES

The problem of taxonomy of Verticillium

The genus Verticillium was erected in 1816 by Nees von Essenbeck on the basis of the production of erect, branched conidiophores with terminal branches (phialides) arranged in whorls. Conidia were unicellular, hyaline or slightly coloured and varied in shape from globose to elliptical (Issac. 1967).

Five plant pathogenic species are now recognised in temperate regions (V.albo-atrum Rienke & Berthold, V.dahliae Klebhan, V.nigrescens Pethybridge, V.tricorpus Issac and V.nubilum Pethybridge), all of which are vascular pathogens (C.M.I., 1970). Because of their wide host range (Englehard, 1957) and economic importance, the taxonomy of these species has been extensively studied. All five pathogens produce 'resting structures' (chlamydospores, microsclerotia or thick walled resting mycelium) which serve as the principle taxonomic criteria for distinguishing the species. Supplementary taxonomic criteria include host range (Issac, 1949), response to temperature (Issac, 1953) and electrophoretic banding patterns of mycelial extracts (Hall, 1969; Milton et al, 1971). Both V.albo-atrum and V.dahliae are morphologically variable and their separation into distinct species is controversial(Buckley et al, 1969; Fitzell et al, 1980; Fordyce and Green, 1964; Hastie, 1973; Issac, 1949; Pelletier and Aubé, 1970; Presley, 1941).

In contrast to the wilt pathogens, the taxonomy of the non-vascular pathogenic and saprophytic species of Verticillium has

received relatively little attention. The genus Verticillium is morphologically similar to the genera Cephalosporium (Corda), Hyalopus (Corda) and Acrostalagmus (Corda). Although there is general agreement that the separate status of the latter two genera is doubtful (Issac, 1967), it is uncertain to which genus they should be referred; Petch (1925) considered both genera synonymous with Cephalosporium whilst Gams (1971) referred them both to Verticillium.

The morphological criteria for the inclusion of an isolate within Cephalosporium are a lack of distinct conidiophores, irregular branching of the aerial mycelium and the occurrence of numerous phialides borne laterally on the hyphae either singly or occasionally in whorls (Balazy, 1973). Gams (1968) also stressed the importance of the septation at the base of the phialide, the affinity of phialides for stains, spore size and shape and the diameter of the hyphae.

Verticillium is currently characterised by the possession of more or less upright distinct conidiophores with whorled phialides, although single phialides may also be present (Gams, 1971; Issac, 1967).

These descriptions allow a considerable overlap in morphological characters between the genera. In his book 'Cephalosporium-artige Pilze' Gams (1971) attempted a coherent treatment of this difficult group of fungi. The genus Cephalosporium was replaced by the older generic name Acremonium (Link ex Fries sensu Gams) which was expanded to include species of Paeciliomyces Bain., Gliomastix Guéguen and several conidial states of Nectria Fr. The genus Verticillium was split into two sections, Erecta and Prostrata, on the basis of conidiophore habit. The former largely corresponded to the original description of the genus made by Nees von Essenbeck, whilst the latter was characterised by occasional whorls of phialides borne on more or less prostrate conidiophores. Several species of Cephalosporium were

transferred to the section Prostrata. These were mainly entomopathogenic species, several of which were also frequently associated with other fungi, eg. V.lecanii (Zimm. Viegas) and Verticillium insectorum. Two species associated with cultivated mushrooms (V.lamellicola (F.E.V. Smith) W.Gams and V.psalliotae (Treschow) were also included in this section.

Several of these species transferred to the section Prostrata were morphologically variable; V.lecanii sensu Gams, for example, was considered synonymous with 23 previously described species. Four new species and 8 new combinations were described within the section, although in some cases the criteria separating the species were scant (eg. V.fusisporum from V.psalliotae).

In a study of the entomopathogenic Cephalosporia (which included many of the species transferred to Verticillium by Gams), Balazy(1973) emphasised the great variation in conidium and phialide size and shape within a single isolate and demonstrated that cultural and environmental factors could have marked effects on morphology. For example, cultures grown at 60 to 90% r.h. produced a greater number of whorled phialides than at 40 and 60%. Phialide arrangement and size and conidial size were also influenced by culture age. Balazy stressed that conidial size alone is an unsatisfactory taxonomic criterion, thus supporting the conclusions of Issac (1967). However, the proportion of spores of different sizes from a single isolate was considered to be of some taxonomic value.

Balazy divided the entomopathogenic species of Cephalosporium into two groups on the basis of microstructure. Five of the isolates studied corresponded to the subgenus Eu-Cephalosporium (Ciferri), whilst the remaining 78 isolates showed characters intermediate between Verticillium and Cephalosporum. It was suggested that the removal of

the entomopathogenic strains of Cephalosporium to the Prostrata section or Verticillium was not justified on morphological grounds and the retention of these species within Cephalosporium was advocated. However, since this group included species with similar morphology and ecological habitats (ie. insects and fungi) and thus formed a reasonably homogenous series, Balazy proposed including them in a new subgenus of Cephalosporium, Pseudoverticillium subgen. nov. This was defined by the absence of distinguishable conidiophores (cf. the description of Cephalosporium above) but with fertile hyphae bearing whorled rather than solitary phialides. C.lecanii Zimm. was cited as the type species. Pseudoverticillium and Verticillium section Prostrata were therefore distinguished by the possession of defined conidiophores by the former. In practice however, the distinction between prostrate, differentiated conidiophores and phialide-bearing hyphae is imprecise. Furthermore. earlier descriptions of several species included in both Pseudoverticillium by Balazy and Verticillium section Prostrata by Gams indicate that they also possess erect conidiophores. For example, C.lecanii was described by Petch (1925) thus: "(conidiophores) arise from repent mycelium .... and stand erect or oblique".

Many single isolates in the present study also produced both erect and prostrate conidiophores and therefore fell between the two sections described by Gams (1971) but could not be included within Pseudoverticillium because of the obvious differentiation of some of the conidiophores.

The confused taxonomy of species of Verticillium which are morphologically and ecologically similar to V.fungicola is further compounded by the lack of information on species included in the section Erecta apart from the vascular plant pathogens. Gams (1971) described two species in this section, Verticillium rexianum (Sacc.)Sacc. and

V.fungicola, which thus leaves an incomplete taxonomic picture of the genus as a whole.

In an attempt to establish satisfactory species limits for V.fungicola and morphologically similar species, isolates of Verticillium obtained during this study were sent to the Commonwealth Mycological Institute, Kew, England (C.M.I.) and the Centraalbureau voor Schimmelcultures, Baarn, The Netherlands (C.B.S.).

Nineteen isolates from peat, soil and spore traps were sent to the C.M.I. On the basis of colony characters the isolates were split into three groups;

(i) Those forming 'felted' colonies producing conspicuous, erect, verticillately branched conidiophores with little aerial mycelium and which were considered to be typical of V.fungicola (Preuss) Hassebrauk.

(ii) Those forming 'cottony' cultures in which the conidiophores were less easily distinguishable from the vegetative hyphae but which also bore numerous verticillate phialides. Several isolates in this group produced a felted mat of erect conidiophores on the agar surface which was overgrown by an aerial mass of cottony mycelium and prostrate conidiophores.

(iii) One isolate produced a dense cottony mass of aerial mycelium and prostrate conidiophores and a deep red colouration of the agar.

Despite this considerable morphological diversity, all the isolates within groups (i) and (ii) were included within V.fungicola on the basis of culture appearance and spore and phialide size and shape. The single isolate in group (iii) was referred to V.psalliotae Treschow on the basis of spore characters and pigment production.

Twenty five isolates were sent to Dr. W. Gams at the C.M.I. including six of those sent to the C.B.S. All of the isolates were included in the section Prostrata despite the frequent occurrence of

erect conidiophores characteristic of the isolates included in group (i) of the CMI identification. The inclusion of these isolates within this section was based on thin conidiophore walls and the tendency for the conidia to take a transverse position at the phialide tip. The inclusion of such isolates with obvious, erect conidiophores within the section Prostrata was a source of considerable confusion in attempts to identify isolates to species level.

The isolates were referred to five species; V.lecanii (Zimm)Viegas, V.bulbillosum W.Gams and Malla, V.lamellicola (F.E.V.Smith)W.Gams, V.leptobactrum W.Gams and V.psalliotae Treschow. One isolate with atypical sporulation was not classified. Isolates corresponding to the group (i) of the CMI were generally included within V.lecanii although one felted isolate was referred to V.psalliotae and a second to V.bulbillosum. Isolates characteristic of group (ii) of the CMI were referred to both V.lecanii and V.bulbillosum.

The inclusion of twelve of the isolates within V.lecanii and V.psalliotae was considered by Gams to be unsatisfactory as it indicated an extraordinary variability within species according to his current species concept, although Domsch, Gams & Anderson (1980) conclude that V.psalliotae "is probably a somewhat heterogenous taxon and comprises of isolates with widely differing conidial measurements".

Two isolates were included within V.bulbillosum by Gams despite the absence of normally characteristic dictyochlamydospores and were viewed as intermediates between this species and V.psalliotae. The occurrence of intermediates between these two species was confirmed by the single isolate in group (iii) of the CMI which possessed all the colony and microscopic characters of a typical V.psalliotae from mushrooms but which also produced abundant dictyochlamydospores. The separation of V.psalliotae and V.bulbillosum on morphological grounds alone therefore seems questionable.

One mushroom pathogenic isolate of V.fungicola was tentatively included within V.lecanii by Gams which underlines the morphological similarities between and difficulties in separating these two species.

I examined the morphology of a further 126 isolates of Verticillium obtained from natural substrates during the course of the study. The characters investigated were: colony type, pigment production, number of phialides per whorl, size and shape of phialides and conidia and the presence or absence of dictyochlamydospores. All the isolates were examined after 7 d growth at 20° on PDA and microscopic preparations were mounted in lactophenol and examined under phase contrast.

In addition, four isolates of V.fungicola from mushrooms were similarly compared and the number of conidia produced cm^{-2} of colony surface was also determined. Discs, 11 mm in diameter, were cut from colony margins and were shaken in 1 ml of sterile distilled water for 2 min. The conidial concentrations were measured using a haemocytometer. Five replicate counts were made per isolate and the results are shown in Table 16.

Of the morphological characters examined, only the presence of dictyochlamydospores proved to be of diagnostic taxonomic value. These structures are characteristic of 3 species in the section Prostrata, viz. V.bublillosum, V.catenulatum (Kamyschko ex Barron and Onions) W Gams and V.chlamydosporium Goddard. In this study the individual species were only occasionally distinguished. Furthermore, as Gams has indicated, the absence of dictyochlamydospores does not necessarily exclude an isolate from these three species.

The other characters examined were variable, and spore size and shape varied considerably within a single isolate. There was also considerable morphological variation between the isolates of V.fungicola examined, this being most marked in colony morphology and sporulation (Table 16).

Table 16. Morphological characters of 4 isolates of V.fungicola obtained from diseased mushrooms. The measurements were made after 7 days' growth on PDA at 20°C and the preparations were mounted in lactophenol.

	Isolate			
	G3	G4	B1	C1
Colony appearance	Cottony	Felted	Centre cottony, margin felted	Cottony
No. phialides per whorl	1-4	1-5	1-3	3-5
Mean *	2.2	2.2	1.8	4.0
Phialide size (um)	14-22 x 1-1.5	14-22 x 1-1.5	18-26 x 1-1.5	18-22 x 1.5-2.0
Mean *	18.4 x 1.4	17.7 x 1.1	20.6 x 1.2	18.8 x 1.7
Conidial size (um)	4-8 x 1.5-2.0	3.5-7.5 x 1-2	3-5 x 1-2	2-8 x 1.5-2.0
Mean @	6.3 x 1.8	5.1 x 1.8	4.3 x 1.5	4.5 x 1.6
Conidial shape	± curved	cylindrical/ elliptical ± pointed ends	subglobose/ elliptical rounded ends	cylindrical/ elliptical rounded ends
No. conidia cm^{-2} colony surface x 10^5	2.0	84.2	7.5	0.2

* Mean of 6 randomly chosen phialides

@ Mean of 10 randomly chosen conidia

Balazy (1973) concluded that Gams' attempt to rationalise and simplify the taxonomy of Cephalosporium like fungi has lead to "a certain number of, perhaps, too far reaching generalisations". The occurrence of intermediates both between the sections and species of Verticillium, the absence of satisfactory limits for V.fungicola and other species and the morphological variability of the isolates examined rendered Gams' taxonomic system for Verticillium of little use in the present study. Consequently, few isolates were identified to the species level. Isolates corresponding to the CMI group (i) were tentatively referred to V.lecanii and other species were only tentatively identified when there was good correspondence with Gams' published description.

Since the accurate identification of organisms is the sine qua non of ecological studies and morphology was of limited use in distinguishing different species of Verticillium, a practical approach to identification was necessary. The sole criterion for the inclusion of an isolate within V.fungicola in this study has been the pathogenicity of the isolate towards cut or growing mushrooms.

Over 300 isolates of Verticillium were obtained, which necessitated the development of a rapid and convenient screening technique.

Pathogenicity tests

Materials and Methods

In vitro test 1. Cut sporophores were inoculated with drops of conidial suspensions of 30 isolates from peat, soil and leaf litter. Suspensions were prepared as described in General Methods. Approximately 50 µl of each suspension, containing 10^5 - 10^6 conidia ml^{-1} were placed on two sporophore pilei. Three pathogenic isolates of V.fungicola and one isolate each of V.psalliotae and V.lecanii were included for

comparison. Controls consisted of sporophores treated with sterile distilled water. The sporophores were incubated in humid chambers at room temperature and were examined after 24 and 72 h.

In vitro test 2. The following test was performed on the majority of isolates, for reasons described in the Results.

Conidial suspensions were prepared and the concentration of conidia determined with a haemocytometer. The suspensions were serially diluted with sterile distilled water to give approximately 2,000 conidia ml^{-1}. Several 50 µl drops of the suspensions were pipetted onto the surfaces of freshly picked sporophores (growth stage 4 - 5) and, in addition, 0.5 ml were also injected into the gill cavity of 2 upturned sporophores (growth stage 3). The sporophores were incubated in humid chambers at room temperature as previously described. Each test consisted of 12 unidentified isolates and, because different strains of mushrooms were used in the different tests, a V.fungicola isolate was included in each test to check for possible variations in symptoms and pathogenicity.

Control sporophores were treated with sterile distilled water. After 7 days, the sporophores were examined for the presence of cap lesions, growth on the gills and/or stipe, collapse of stipe tissue and distortions of stipe apex tissue. A scoring system was devised to summarize these results in which a score of 2 was given for each of the five characters present, 1 for each character present but poorly manifested and 0 if the character was absent. The maximum score possible was therefore 10.

RESULTS

In vitro test 1.

After 24 h the three *V.fungicola* isolates had produced sunken lesions lined with dense, erect conidiophores. Although the response of these isolates was the most rapid, 9 other isolates (including *V.lecanii* and morphologically similar isolates) produced similar symptoms. Twenty five of the 30 isolates caused tissue collapse associated with a cottony aerial mycelium with verticillate conidiophores. *V.psalliotae* produced only slight tissue collapse and little aerial mycelium and this may reflect the higher optimum temperature required by this pathogen.

Since many of the isolates tested produced some symptoms that might be confused with those produced by *V.fungicola*, an attempt was made to alter the assay conditions to increase specificity. When a serial dilution of *V.fungicola* G3 conidia was applied to sporophore cap tissue and was incubated for 3 days, pitting of the tissue and the development of mycelium and conidiophores occurred at inoculum concentrations as low as 300 conidia ml^{-1} (Plate 50). With *V.lecanii* C3 however, no symptoms were produced below 2,000 conidia ml^{-1} (Plate 51).

Further tests using 5 isolates of *V.fungicola* and isolates of *V.lecanii*, *V.psalliotae* and *V.bulbillosum* indicated that the production of browned, sunken lesions lined with felted aerial conidiophores was specific to *V.fungicola* when low concentrations of inoculum were used. It was also established that only isolates of *V.fungicola* produced a dense, felted growth on gill and stipe tissue and occasional distortions of stipe apex tissue when dilute inocula were injected into gill cavities through unopened veils. This method of inoculation also had the advantage that contaminating microorganisms were excluded. The majority

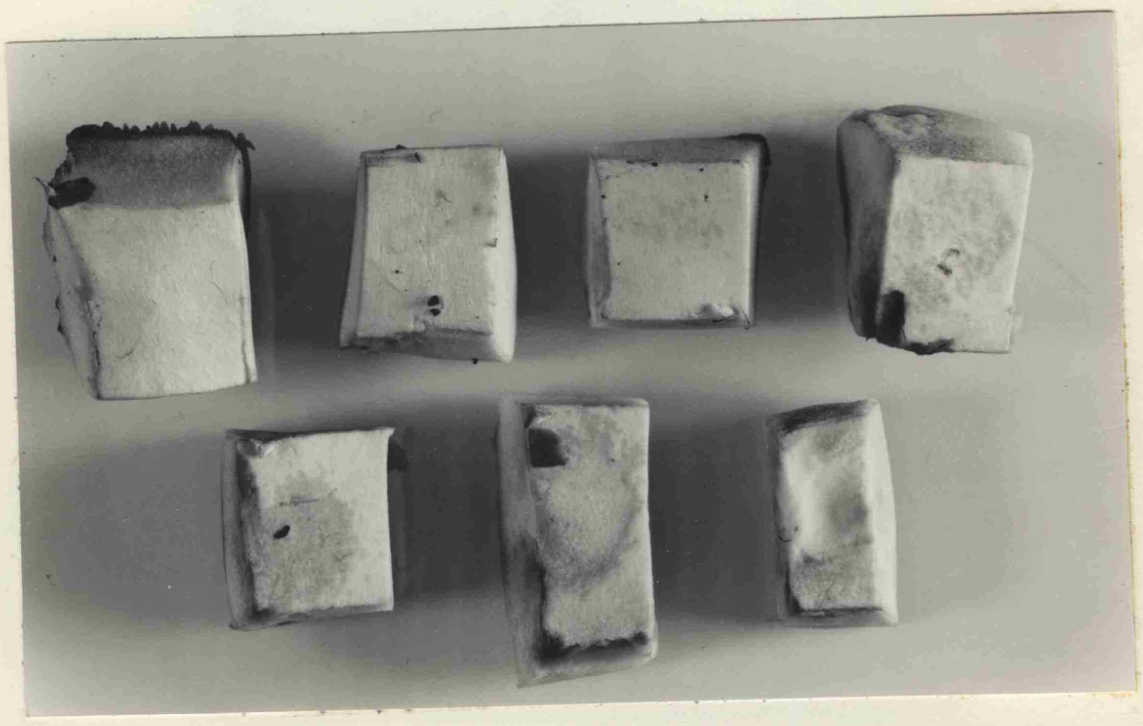


Plate 50 Symptoms produced on mushroom cap tissue 3 d after inoculation with a serially diluted V.fungicola isolate G3 conidial suspension. Conidial concentrations (ml^{-1}) were:-

Top row, left to right, 0 , 3.4×10^{1}, 3.4×10^{2}, 3.4×10^{3}.

Bottom row, 3.4×10^{4}, 3.4×10^{5}, 3.4×10^{6}.

The tissue blocks were incubated at 20°C in humid chambers.

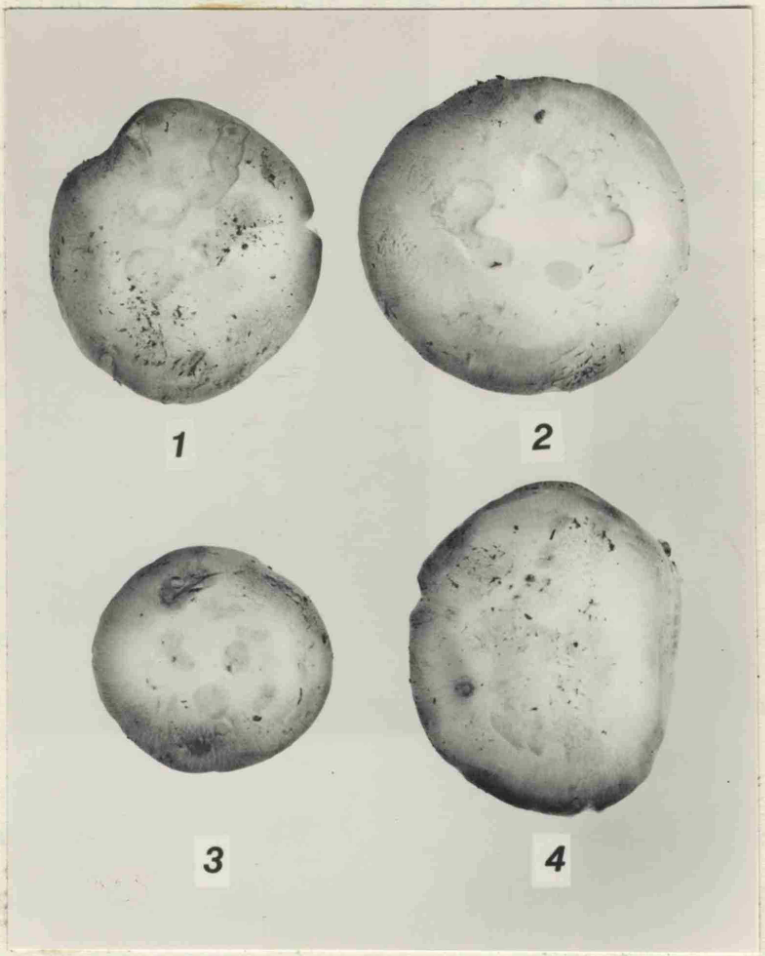


Plate 51 Symptoms produced on mushroom caps 3 d after inoculation with a serially diluted V.lecanii isolate C3 conidial suspension. Conidial concentrations (ml^{-1}) were:-

1. 2×10^6 2. 2×10^5 3. 2×10^4 4. 2×10^3.

The sporophores were incubated at 20°C in humid chambers.

of isolates were therefore subjected to test method 2.

In vitro test 2.

The scores obtained by isolates of Verticillium identified by morphological characters are given in Table 17. All the V.fungicola isolates consistently scored at least 8 and this was taken as the minimum required to define an isolate as pathogenic. V.psalliotae isolated from mushrooms scored 7, but other isolates morphologically identical as well as other species morphologically distinct gave variable scores. The significance of scores between 0 and 7 in terms of pathogenicity is therefore uncertain.

Of the 278 isolates tested from the 6 habitats examined, only 3 scored 8 or above (Table 18). These were isolate 3J 13 from a soil sample taken at the GCRI turf site, August, 1980, isolate LL 19 3 from deciduous wood leaf litter, Horton, Avon, April 1980 and isolate CH 1 6 from crushed limestone, February 1980. None of these isolates were morphologically identifiable as V.fungicola. Within each habitat group, except for the fly traps and crushed limestone from which few isolates were recovered, isolates produced scores ranging between 0 and 7, with 0 being the most common. Many of the isolates initially appeared to have no distinguishing features but were subsequently shown to produce dictyochlamysospores.

Pathogenicity field tests

Although the tests described above gave a rapid and convenient screen for pathogenicity, it was important to determine whether the reaction of isolates on cut sporophores was a true indication of pathogenicity ie. whether any of the isolates could also reproduce the entire disease syndrome on growing sporophores.

Table 17. The reaction of six species of Verticillium in the in vitro pathogenicity test 2. 0 = not pathogenic, 10 is the maximum score and 8 is taken as the minimum score for pathogenicity.

Species	Isolate	Score
V.fungicola	G3	8-9
"	G4	8
"	G5	8
"	C1	8-10
"	S1	8
"	S2	8
V.psalliotae	C9	7
V.lamellicola	M1	0
V.lecanii	C3	0
V.tricorpus	M1	0
V.lateritium	S1	0

Fifty-two isolates (29 from peat, 9 from soil, 7 from leaf litter, 3 from spore traps, 1 V.lecanii from insects (C3), 1 V.psalliotae from mushrooms and 2 V.fungicola (G3 and G4) from mushrooms) were tested by inoculating beds of growing mushrooms with conidial suspensions under conditions conducive to the development of dry bubble.

Materials and Methods

The experiment was conducted in the cropping chambers of the mushroom isolation unit at the GCRI. Two chambers were used, each containing 16 wooden trays of 0.56 m^2 surface area.(Plate 52). The trays were filled with a synthetic compost (GCRI Formula 2; Randle,1974)

Plate 52 Mushroom growth chamber used for the field pathogenicity test. Note the plastic sheet dividing each tray and the capillary watering system which reduced the risk of cross contamination of the isolates.

which is based on wheat straw to which water, chicken manure, molassed brewer's grain and gypsum are added during composting. The compost was spawned with A.bisporus strain Darlington 621 and was spawn run and cased in the normal way. After casing, the cropping area of each tray was divided in half using 15 cm high strips of 500 gauge polythene sheeting which was supported with plastic strips. A capillary watering system was installed (Flegg, 1962) using strips of 3 cm wide capillary matting which were laid across the casing surface with one end dipped into containers of clean water. Both these measures minimised the risk of cross contamination of the plots by water splash. Occasionally, additional watering was necessary and care was taken to keep water splash to a minimum.

The beds were inoculated 10 days after casing (7/7/80). Conidial suspensions of each isolate were prepared from four 10 day old cultures on PDA, the volume being made up to 125 ml with sterile distilled water. Most of the resulting suspensions were turbid and measurement of the conidial concentrations of two of the least turbid suspensions showed that they contained at least 10^5 conidia ml^{-1}. The suspensions were sprinkled over the half trays to which they had been randomly designated, ensuring that no cross contamination of the suspensions took place. To maximise the number of isolates tested, only one half tray plot was inoculated per isolate. Six control plots in each chamber were treated with sterile distilled water.

The chamber temperature was maintained at 17°C and the r.h. between 85 and 95% throughout cropping. Mushroom flies were kept to a minimum using sprays of the synthetic pyrethroid 'Pynosect' and mites were controlled with sprays of 'Kelthane'. The mushrooms were harvested as recommended for commercial practice and hands and knives were rinsed in alcohol between each plot. The numbers and weights of healthy and

diseased sporophores were recorded, the latter being classified as spotted, distorted and sclerodermoid. The last harvest was made on 11/8/80.

RESULTS

The results of the 'field' pathogenicity test are summarised in Table 19, in which the numbers and weights of diseased sporophores expressed as a percentage of the total yield have been categorised and the frequency of isolates within each category is shown.

The majority of isolates produced below 5% diseased sporophores. Five isolates produced over 10% diseased sporophores and the identity of these is indicated in the Table. The 2 V.fungicola isolates produced the greatest numbers of diseased sporophores, isolate G4 yielding over twice as many (70% of the total number) as isolate G3 (34% of the total number). On a weight basis however the proportions of diseased sporophores for these two isolates were 54% and 36% of the total yield respectively. The other 3 isolates which produced over 10% diseased sporophores could all be included within V.lecanii (C.M.I. group 1 - see page 88).

The percentage by number of diseased sporophores in each of the three disease classes (spotted, distorted and sclerodermoid) for the 5 isolates which produced over 10% disease is shown in Table 20. Only the V.fungicola isolates produced the whole range of disease symptoms, isolate G4 producing over twice as many distorted and sclerodermoid sporophores as G3. This may be due to differences in pathogenicity between the isolates but probably also reflects differences in the concentration of inoculum, since isolate G4 sporulates more abundantly on agar media than isolate G3. Distorted and sclerodermoid sporophores were also produced on the V.lecanii C3 plot but these were due to contamination from the V.fungicola G4 plot directly above.

Table 19. Summary of the results of the field pathogenicity experiment. The yield of diseased sporophores as a proportion of the total yield on both a number and weight basis has been categorized into 10 groups and the number of isolates in each disease category is shown. All 12 control plots showed some symptom development but this was largely due to contamination by a *Penicillium* species (see text).

Proportion of total yield (% by number, weight) diseased.	Percentage of isolates in each disease category.		Identity.	Number of control plots in each category.	
	By number.	By weight.		By number.	By weight.
0 - 10	47	46		12	12
11 - 20	2	3	*V.lecanii* C3/P94	0	0
21 - 30	1	1	*V.lecanii* ST29	0	0
31 - 40	1*	1*	*V.fungicola* G3	0	0
41 - 50	0	0		0	0
51 - 60	0	1	*V.fungicola* G4	0	0
61 - 70	1	0		0	0
71 - 80	0	0		0	0
81 - 90	0	0		0	0
91 - 100	0	0		0	0

* Mixed *Verticillium* and *Penicillium*.

The results of the experiment were complicated by the occurrence of a parasitic Penicillium species which caused a brown spotting of the sporophore cap very similar to that produced by V.fungicola (Plate 53). This appeared in the first flush and spread rapidly and was initially confused with the symptoms of dry bubble cap infections.

In an attempt to determine which pathogen was causing the symptoms, isolations were made from the lesions as the experiment progressed. Both Penicillium and Verticillium were isolated from the same plots (Table 21) and frequently from the same sporophore.

Table 20. Type of symptom produced by 5 isolates in the field pathogenicity experiment which resulted in more than 10% diseased sporophores.

	Percentage of the total number of diseased sporophores within each disease category.		
Isolate	Spotted	Distorted	Sclerodermoid
V.fungicola G4	14.9	41.5	43.2
V.fungicola G3	68.1	16.6	15.1
V.lecanii ST 29	100.0*	0.0	0.0
V.lecanii C3	71.7+	15.2+	13.0+
V.lecanii P94	100.0*	0.0	0.0

* Mixed Verticillium and Penicillium

\+ Directly below V.fungicola G4 and therefore probably due to cross contamination.

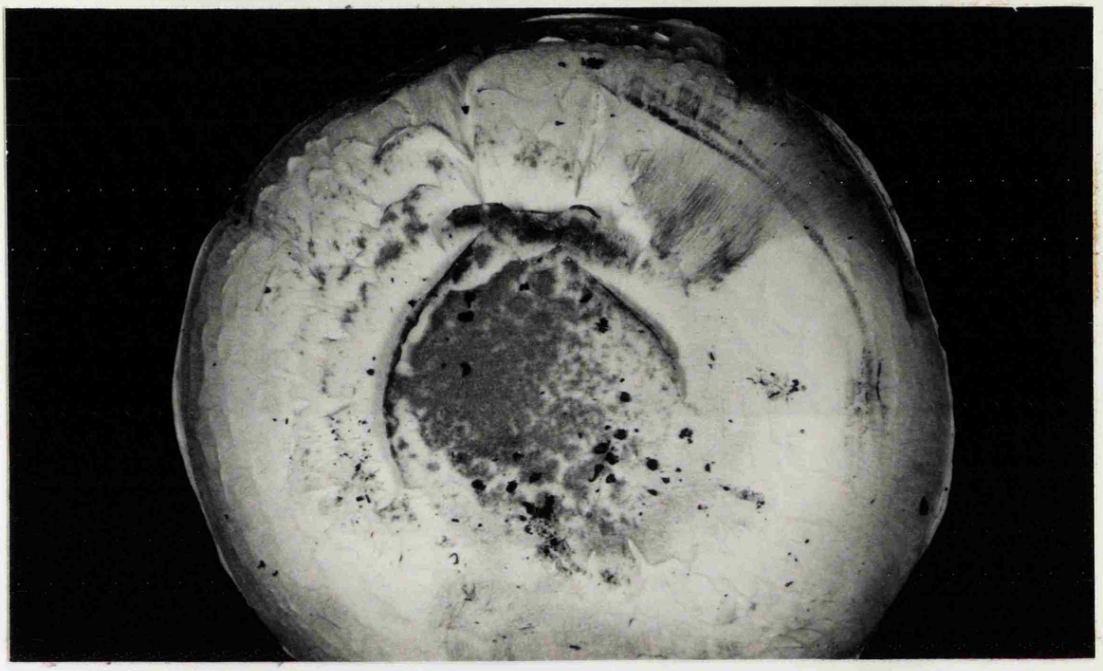


Plate 53 Symptoms caused by the pathogenic Penicillium sp. that occurred in the field pathogenicity test. Prior to the production of aerial sporulation mycelium on the mushroom cap, the symptoms were similar to those produced by V.fungicola.

Table 21. Summary of the results of isolations of *Verticillium* & *Penicillium* from plots in the field pathogenicity experiment.

	No of plots from which *Penicillium* was isolated	No of plots from which *Verticillium* was isolated	No of plots from which both were isolated
Inoculated plots	44	35	35
Control plots	12	4	4

Attempts to reproduce the *Penicillium* disease by artificial inoculation of cut sporophores with several of the *Penicillium* isolates were unsuccessful. It was not possible to determine satisfactorily the proportion of spotting due to *Penicillium* and that due to *Verticillium* in each plot but many of the disease records within the 0 - 10% category are likely to be due to *Penicillium* alone. Also, the large proportions of diseased sporophores recorded on the *V.lecanii* P 94 and ST 29 plots were probably in part due to *Penicillium* but it is interesting that these isolates, which are morphologically most similar to *V.fungicola*, should produce the greatest amount of cap spotting. In the absence of replication and statistical analysis the significance of these results remains uncertain.

The morphology of the isolates of *Verticillium* recovered from sporophore lesions on each plot was also compared with the isolates originally applied to the plots. Twenty of the 35 isolates recovered corresponded with the initial inoculum whilst the others were probably contaminants from the casing.

In spite of the precautions to prevent the spread of dry bubble,

cross contamination did occur. This was first observed after 3 weeks as distorted sporophores in trays adjacent to the 2 V.fungicola isolates and the disease continued to spread until the experiment was terminated after 5 weeks. All the isolates of V.fungicola obtained from the control plots were V.fungicola contaminants.

Discussion

When compared with results from the field pathogenicity test, the results of the in vitro test were variable. The most complete results were those of the peat isolates which are shown in Table 22. None of the isolates tested produced disease in more than 6% of the total number of sporophores harvested in the field assay but the scores in the in vitro test for the same isolates ranged from 0 - 7. This suggests that this score range does not indicate a progressive increase in pathogenicity and reinforces the suggestion that the in vitro test may only be useful on a qualitative basis, ie. isolates with scores of 8 and above may be considered pathogenic whilst isolates with scores below 8 may not.

Development of a selective medium

In studying the ecology of a particular microorganism it is important to be able to isolate it consistently from the environment and one of the most convenient ways to achieve this is with a selective medium. Many such media have been devised for the isolation of plant pathogenic fungi from natural substrates, including plant pathogenic Verticillium species (Tsao, 1970). In media devised for the isolation of some Verticillium species, selectivity depends on the enhancement of production

Table 22. Comparison of the *in vitro* pathogenicity test scores with the field pathogenicity test for the peat isolates.

Percentage of the total number of sporophores diseased.

Isolate	In vitro test	In the field pathogenicity test	Tentative identity
P94	0	2.3	*V.lecanii*
P154	0	1.0	D.
P176	7	0.6	*V.lecanii*
P183	0	2.3	D.
P186	0	5.7	D.
P215	0	5.1	*V.lecanii*
P219	4	1.5	*V.psalliotae*
P271	4	0.0	*V.lecanii*
P276	0	1.0	D.
P282	2	3.4	*V.lamellicola*
P283	4	0.0	*V.lecanii*
P328	0	0.2	D.
P331	7	2.6	?
P337	0	0.0	D.
P338	5	5.5	*V.lecanii*
P353	5	2.0	*V.bulbillosum*
P371	0	4.9	?
P392	0	2.9	?
P393	2	0.0	?
P402	0	1.4	D.
P407	3	0.6	?

D. = dictyochlamydospores present
V.lecanii = isolates which produced felted mycelium with frequent erect conidiophores (= C.M.I. group 1; see page 88.)
* This isolate was morphologically intermediate with *V.psalliotae* (= C.M.I. group III; see page 88.)
? Not identified

or visibility of microsclerotia or other resting structures, whilst fast growing contaminants are suppressed with antibiotics and fungicides (Evans et al, 1967; Jordan, 1971; Nadakavukaren & Horner, 1959).

Materials and Methods

Several complete selective media devised for *Verticillium* and other soil fungi (Table 23) were examined first.

Table 23. Complete selective media tested for growth of *V.fungicola* and *Trichoderma viride*.

Selective medium	Selective principle	Selective for
Nadakavukaren and Horner (1959)	Ethanol, PCNB	*V.dahliae*
Jordan (1971)	Sorbose	"
Nash and Snyder (1962)	PCNB, streptomycin	*Fusarium* spp.
VP Tsao and Ocana (1969)	PCNB, vancomycin, pimaricin	*Pythium* spp.
Oxgall medium (Littman, 1947)	Oxgall, crystal violet	Fungi in general

Gandy (unpublished data) established that *V.fungicola* grows on a wide range of carbon and nitrogen sources and developed a simple defined medium that supports rapid linear growth. A modification of this medium, with the mushroom storage carbohydrate mannitol as carbon source and buffered to decrease the growth of contaminant fungi was chosen as the basic medium for the evaluation of potential selective agents.

Basal Medium

mannitol	BDH	15.0 g
KNO_3	BDH	1.0 g
$NaHPO_4$	BDH	15.5 g
citric acid	BDH	1.5 g
agar (Oxoid No 3)		15.0 g
distilled water		1 L

pH 7.2 after autoclaving 15 min at 120°C

Potential selective agents (Table 24) were incorporated into this medium as stock solutions, giving final concentrations of 10 and 100 µg ml^{-1}. In the controls, the volume was made up with sterile distilled water.

Petri dishes containing the media were inoculated with mixed conidial suspensions (total concentration $2x10^5$ ml^{-1}) and with discs of mycelium of V.fungicola isolate G3 and T.viride isolate T1 obtained from soil. T.viride was included in the tests because it is a fast growing species commonly found in soil and other substrates and was thus likely to occur as a contaminant on isolation plates. The relative development of the two fungi was examined after incubation for 7 days at 20°.

In further tests to determine the maximum concentrations of PCNB and copper sulphate in the basal medium that would permit the growth of V.fungicola and which would therefore be most likely to repress the growth of contaminants, the concentration of PCNB was varied from 10 - 1000 µg ml^{-1} and copper sulphate from 100 - 6000 µg ml^{-1}. Plates of the media were inoculated with 0.5 ml of a V.fungicola conidial suspension (c. 10^5 ml^{-1}) which was spread over the agar surface with a bent glass rod. The plates were incubated in the dark at 20° and periodically examined .

Table 24. Potential selective agents incorporated into basal medium at 10 and 100 µg ml^{-1} (except where otherwise stated).

Class of substance	Name
Antibacterial antibiotic	Chlorotetracycline
	Streptomycin
	Novobiocin
	Polymixin B sulphate
Antifungal antibiotic	Pimafucin
Fungicides	Thiram
	Daconil
	Zineb
	Pentachloronitrobenzene (PCNB)
	Copper oxinate
Surface active agents	Tween 20 (non-ionic)
	Sodium dodecyl sulphate (anionic)
Chelating agent	8 hydroxy quinoline
General growth inhibitors	Rose bengal
	Copper sulphate +

+ 10, 100 and 1000 µg ml^{-1}

The efficiency of recovery of V.fungicola from conidial suspensions was determined using the final form of the selective medium (SM1).

The PCNB, copper sulphate and aureomycin were added to the molten medium (c. 50°C) after autoclaving as stock solutions in 15 ml of sterile distilled water. The medium was thoroughly mixed after the addition of each chemical and 20 ml amounts were dispensed into Petri dishes.

Selective Medium SM1

Mannitol (BDH)	15.0 g	
KNO_3 (BDH)	1.0 g	
Na_2HPO_4 (BDH)	15.5 g	
citric acid (BDH)	1.5 g	
agar (Oxoid No 3)	15.0 g	
distilled water	1 l	
20% PCNB (Brassicol 20, Hoechst)	2.5 g	(= 500 $\mu g\ ml^{-1}$ ai)
copper sulphate (BDH)	3.0 g	(=3000 $\mu g\ ml^{-1}$ ai)
Veterinary grade aureomycin (Cyanimid Corp.)	2.6 g	(= 200 $\mu g\ ml^{-1}$ ai)

A conidial suspension of V.fungicola isolate G3 was prepared and diluted to give a range of concentrations from 7.6×10^5 to 7.6 conidia ml^{-1}. The suspensions were incubated at 20° for 24 h and were then serially diluted so that a 0.1 ml aliquot of the final diluted suspension was calculated to contain between c. 8 and 800 conidia. This volume of suspension was pipetted onto duplicate plates of both freshly prepared selective medium and PDA and was spread over the agar surface with a bent glass rod. Two plates of both media were also treated with 0.1 ml drops of sterile distilled water. The plates were incubated at 20° and the numbers of colonies that developed were counted after 3 and 21 days for PDA and the selective medium respectively. The results were expressed as mean percentage recovery which was calculated as the (mean number of colonies observed ÷ number of colonies expected if recovery was 100% efficient) x 100, assuming that each colony arose from a single conidium.

Using medium SM1 attempts were made to recover V.fungicola from artificially infested casing and soil. Duplicate 10 g samples of casing or soil (a clay - loam, pH 6.5) were infested with 1 ml of a washed

V.fungicola isolate G3 conidial suspension giving final concentrations of c. 3×10^5 and 3×10^4 conidia g^{-1} fresh weight of casing and soil respectively. Control samples were treated with 1 ml of sterile distilled water. After incubation at 20° for 24 h the samples were shaken with 100 ml of 0.1% water agar in 250 ml flasks for 10 minutes on a wrist action shaker and the resulting suspensions were serially diluted to give calculated concentrations of between 50 and 200 conidia in a 0.1 ml aliquot of the final dilution. This volume was pipetted onto the surface of 3 replicate plates of freshly prepared selective medium and was spread over the agar surface with a bent glass rod. The plates were incubated in the dark at 20° for 3 weeks and the number of Verticillium colonies recovered was recorded. The experiment was repeated using casing infested with 1.2×10^4 conidia g^{-1} fresh weight of V.fungicola isolate G4 incubated for 24 h, and with 1×10^6 conidia g^{-1} fresh weight of V.fungicola isolate G3 incubated for 0.5 hours. The results were again expressed as mean percentage recovery.

Results

None of the media described in Table 1 proved to be satisfactorily selective for V.fungicola.

The most promising of the anti-fungal agents tested were 20% PCNB [Brassicol 20, (Hoechst)] and copper sulphate (BDH) both of which permitted the growth of V.fungicola at the highest concentration tested whilst completely preventing the growth of T.viride. Both of these substances have been successfully used in a medium selective for the related fungus Cephalosporium gramineum Nisikado & Ikata (Ravenscroft and Wiese, 1972).

Growth and sporulation of V.fungicola on media containing all concentrations of PCNB and $CuSO_4$ was restricted compared with growth

on PDA. A lawn of mycelium developed only after about 3 weeks and, as might be expected, growth was poorest at the highest concentrations tested. However, faster growing sectors were frequently produced.

The recovery of V.fungicola from conidial suspensions using both medium SM1 and PDA is shown in Table 25. The percentage recovery on the selective medium ranged from 59.2 - 86.2 % and from 72.3 - 123.9 % on PDA. The lowest recovery rates coincided with the 2 lowest concentrations of inoculum with both media. At higher inoculum concentrations the efficiency of recovery was generally independent of inoculum concentration for both the media.

The greater recovery rate of inoculum on PDA suggests that the selective medium is to a certain extent toxic. The recovery of a greater number of propagules than expected on PDA is probably due to errors in the preparation and plating of the dilution series. Colonies grew rapidly and sporulated abundantly on PDA whilst on the selective medium growth was restricted and sporulation poor.

In contrast to the reasonable degree of recovery of incoulum from conidial suspensions, the recovery from artificially infested casing and soil was disappointingly variable and poor (Table 26). With isolate G3 the mean percentage recovery from both substrates was similar after an incubation period of 24 h whilst the recovery of isolate G4 was over 6 times as great. Shortening the incubation period of isolate G3 in casing to 0.5 h did not however result in an increase in the amount of inoculum recovered.

V.fungicola was not the only fungus to develop on the isolation plates but the growth of other colonies (mainly Penicillium spp.) was restricted although Cephalosporium and Fusarium spp. occasionally spread across the plates. None of the isolation plates were overrun with contaminants commonly encountered in studies of the fungal flora of soils and other substrates, eg. Mucor and Trichoderma spp.

Table 25. The recovery of V.fungicola colonies from a serially diluted conidial suspension incubated for 24 h at 20°C using both PDA and selective medium SM1. The isolation plates were incubated for 3 days (PDA) and 21 days (SM1) in the dark.

Number of conidia ml^{-1} of original suspension.	Dilution factor	Expected number of colonies per plate.	Mean observed number of colonies per plate.		Mean percentage recovery.	
			PDA	SM1	PDA	SM1
7.6×10^1	0	7.6	5.5	4.5	72.3	59.2
7.6×10^2	0	76	55.0	45.0	72.3	59.2
7.6×10^3	0	760	805.5	651.0	105.9	85.6
7.6×10^4	10	760	857.0	508.5	112.7	66.9
7.6×10^5	100	760	810.5	655.5	106.5	86.2
7.6×10^6	1000	760	942.0	605.0	123.9	79.6
Water control	0	0	0	0	0	0

Table 26. The recovery of V.fungicola from artificially infested casing and soil using the selective medium.

Experiment No.	Substrate/ isolate	Incubation Time (h)	Inoculum Concentration conidia g^{-1} fresh weight	Mean % recovery
1.	Casing G3	24	3×10^5	4.7
2.	Soil G3	24	3×10^4	5.9
3.	Casing G3	0.5	1×10^6	1.0
4.	Casing G4	24	1×10^4	38.4

No Verticillium colonies were recovered from the water controls.

Discussion

The relatively high percentage recovery of V.fungicola from a range of concentrations of conidia in suspension using medium SM1 suggested that it would be of considerable value in the investigation of the ecology of the fungus in natural substrates. However, this was not borne out by attempts to re-isolate V.fungicola from artificially infested casing and soil.

There are several possible explanations for this poor recovery. In the conidial suspension experiment, in which V.fungicola was the only microorganism present, the counts of the small, uniform colonies were easily made. When plates of the selective medium were inoculated with soil or peat suspensions, however, particles of the substrates and colonies of other microorganisms tended to mask the development of V.fungicola and the slow growth and poor sporulation of the colonies rendered identification difficult. Consequently the colony counts may not have been truly representative of the actual number of conidia present. In one of the experiments (isolate G4 in casing) all suspected

V.fungicola colonies were transferred to PDA to check their identity. The majority of these colonies were found to conform to V.fungicola but the overall recovery was still only 40% which suggests that poor colony identification was not the only factor involved in the low percentage recovery.

It is also possible that the conidia lost viability within the soil or casing. However, Cross (1971) demonstrated that conidia may remain viable within moist casing for over 6 months and, although he gave no indication of the numbers of viable conidia remaining after this time, the fact that the infested casing still caused disease when applied to mushroom beds suggests that the proportion was high. The death of over 90% of conidia within 24 h therefore seems unlikely. This is further borne out by the poor recovery of inoculum from casing after an incubation period of only 0.5 hours during which time mortality of the conidia is likely to be very low.

Finally, the poor recovery of V.fungicola may in part be due to a failure to resuspend the conidia after incubation. Although there has been little work on the retention of fungal conidia within soil, both bacteria and streptomycetes are capable of being firmly and irreversibly bound to particles of soil and other solid substrates (Balkwill & Casida, 1979). In many cases, adhesion is thought to be due to an interaction between charged extracellular microbial polysaccharides and surface charges on solids and is particularly well manifested in substrates containing a high proportion of colloidal material, such as peat and clay (Hattori, 1970; Marshall, 1976). V.fungicola conidia are coated with a sticky mucilage and such microbial mucilages are often predominantly composed of polysaccharide (Bracker and Littlefield, 1973) and this, together with the small size and consequently large surface area/mass ratio of the conidia would favour adhesion within soil. The adhesive

properties of V.fungicola conidia are also demonstrated by their very rapid and firm attachment to glass surfaces, a phenomenon that has been frequently observed during the washing of conidia by centrifugation.

Because of adhesion to soil particles, the successful isolation of bacteria from soil usually requires violent agitation (for example, in a Waring blender), sonication, or treatment of the soil suspension with detergents. It is possible that the relatively mild extraction procedure used in these experiments, although of a type recommended for the isolation of soil fungi (Johnson et al, 1959), was insufficiently vigorous to resuspend a large proportion of the conidia. Counts of Verticillium species obtained from dilution plating of soil or peat samples are therefore likely to underestimate the true population, irrespective of the isolation medium used. This problem is made worse on non-selective media because the small number of Verticillium colonies developing is rapidly overrun by faster growing fungi. To minimise this problem the PCNB/copper sulphate medium was used in all the studies of natural substrates described below.

E C O L O G Y

Introduction

Plant pathogenic Verticillium species have a widespread geographical distribution but predominate in temperate regions (CMI, 1970). The epidemiology of V.albo-atrum and V.dahliae has been extensively studied and both species are primarily soil borne pathogens. Using various selective media, both qualitative and quantitative estimates of the inoculum within soil have been made(Ashworth et al, 1972; Ausher et al, 1976; Evans et al, 1967; Harrison & Livingston, 1966; Nadakavukaren & Horner, 1959). In the absence of susceptible hosts, inoculum of V.albo-atrum declines within 2 - 3 years in soil whilst V.dahliae may maintain a high inoculum potential for many years due to the production of microsclerotia (Keyworth, 1942; Schrieber & Green, 1962; Sewell & Wilson, 1966; Wilhelm, 1955). Disease problems are therefore most severe where repeated cropping with susceptible hosts is practised. Weed hosts have been implicated as a means of carry over from crop to crop and may allow a build up of inoculum within the soil (Wilhelm & Thomas, 1952). Non-host plants have also been shown to harbour V.albo-atrum and may constitute a further source of inoculum (Martinson & Horner, 1962).

Infection of host plants from the soil is by penetration of the root cortex or via wounds. The pathogen then systemically invades the xylem causing wilting (Rudolph, 1931). As the infected plant senesces and dies, the pathogen colonises the cortical tissue and produces resting structures which enter the soil as the plant decomposes. Wilhelm (1951) has demonstrated that V.dahliae cannot grow saprotrophically through unammended, non-sterile soil and that conidia and mycelium are short lived in soil. The addition of organic ammendments (eg. plant debris), however, stimulates both growth and sporulation (Powelson & Patil, 1963).

The ecology of other species of Verticillium has received little attention, partly because of their lesser economic importance but also because of the difficulties encountered in their identification. Non-vascular pathogenic species of Verticillium have been recorded in diverse habitats including fungal sporophores, rust fungi, insects, spiders and vertebrates (Gams, 1971), rotifers (Barron, 1973), rock surfaces (Went, 1969), soil and leaf litter of various types (Bååth & Soderstrom, 1980; Bisset & Parkinson, 1980; Christensen & Whittingham, 1965, 1965; Dickinson & Dooley, 1967; Gams, 1971) and leaf surfaces (Darke et al, 1976; Dickinson, 1973).

The sources of isolates of Verticillium species described by Gams (1971) are summarised in Table 27. This shows the trends in ecology within and between the species and suggests possible sources of V.fungicola. The most common sources of isolates were insects (10 species), soil (9 species) and other fungi (8 species).

The species associated with the cultivated mushroom (V.fungicola, V.psalliotae and V.lamellicola) have been isolated from agaric sporophores, rust and powdery mildew fungi, soil, leaf litter and insects. These substrates, as well as horticultural peat and ground limestone (the constituents of casing) were examined as possible sources of V.fungicola using the selective medium described previously.

Substrates investigated

a. Soils

Primary outbreaks of dry bubble disease have often been associated with large scale soil disturbance in the vicinity of mushroom farms, especially during dry weather when dust and debris readily become airborne (Gandy, 1978) and it has long been assumed that soil is a reservoir of V.fungicola inoculum (Kneebone & Merek, 1961). There is however only

Table 27. Sources of *Verticillium* species as summarised by Gams(1971)

	Cultivated mushrooms	Wild fungal sporophores	Rust fungi	Powdery mildew fungi	Myxomycetes	Other fungi	Soil	Leaf litter, wood, humus	Insects, spiders	Living plants	Vertebrates	Nematodes
*V.fungicola**	#	+	+				+	+				
V.psalliotae	+	+	+			+	+		+	+		
V.lamellicola	+	+	+							+	+	
V.lecanii		+	+			+	+	+	+	+		
V.bulbillosum							+	+		+		
V.chlamydosporium							+					+
V.catenulatum							+	+				
V.arenarium									+			
V.cephalosporium							+	+				
V.falcatum									+			
V.fusisporum		+					+		+			
V.griseum									+			
V.hemipterigenum									+			
V.insectorum		+		+			+		+			
V.indicum									+	+		
V.leptobactrum		+						+				
*V.rexianum**					+							
Verticillium sp.1									+			
Verticillium sp.2		+							+			

* Section *Erecta*; all other species, section *Prostrata*.

Verticillium sp. 1 = conidial state of *Cordyceps ophioglossoides*(Ehrenb)Lk
Verticillium sp.2 = conidial state of *Torrubiella alba* Petch

one authenticated record of the pathogen from soil, from Brisbane, Australia (Gams, 1971). V.psalliotae has a wider distribution in soils having been isolated from woodland and lead mine soils in the UK and from wheat field soils, also in Australia (CMI, 1976).

A range of soil types was investigated for the presence and seasonal distribution of V.fungicola and since different methods of isolation are thought to give different qualitative and quantitative estimates of the fungal flora (Chesters and Thornton, 1956; McLennan and Ducker, 1954; Sewell, 1959; Warcup, 1957), the dilution plate technique was supplemented by direct plating of the samples.

Materials and Methods

1. Sample sites

GCRI sites

Five sites approximately 2 x 2 m^2 were chosen for regular sampling at the GCRI (Fig. 20). They were of similar basic soil type (brick earth) but each had a different vegetation cover. The sites were sampled each month from January to November 1980.

Sites other than the GCRI

Samples were also taken from 27 sites in England and Scotland which represented a wide range of soil type and vegetation cover (Fig. 21). The sites were sampled in 1979 and 1980.

2. Sampling and isolation methods

Five approximately 50 g sub-samples were taken from the top 7 cm of soil at each sampling site and were bulked in the field. In the woodland sites care was taken to remove as much leaf litter as possible before sampling, but contamination of the soil with litter was common. All the samplings were performed with alcohol sterilised implements and

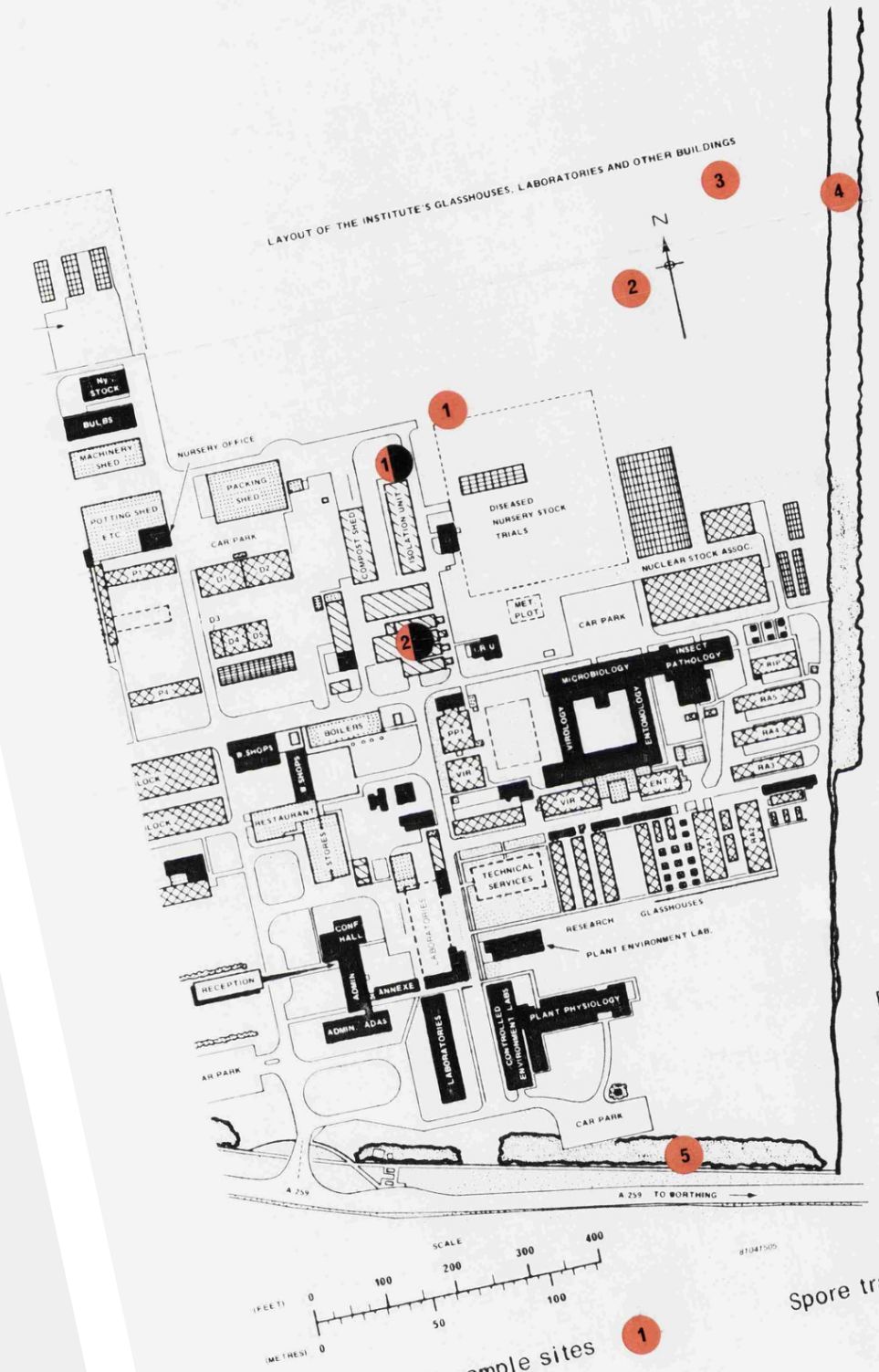


Fig.20. Soil sample and spore trap sites at the GCRI. The figures refer to the key on the following page.

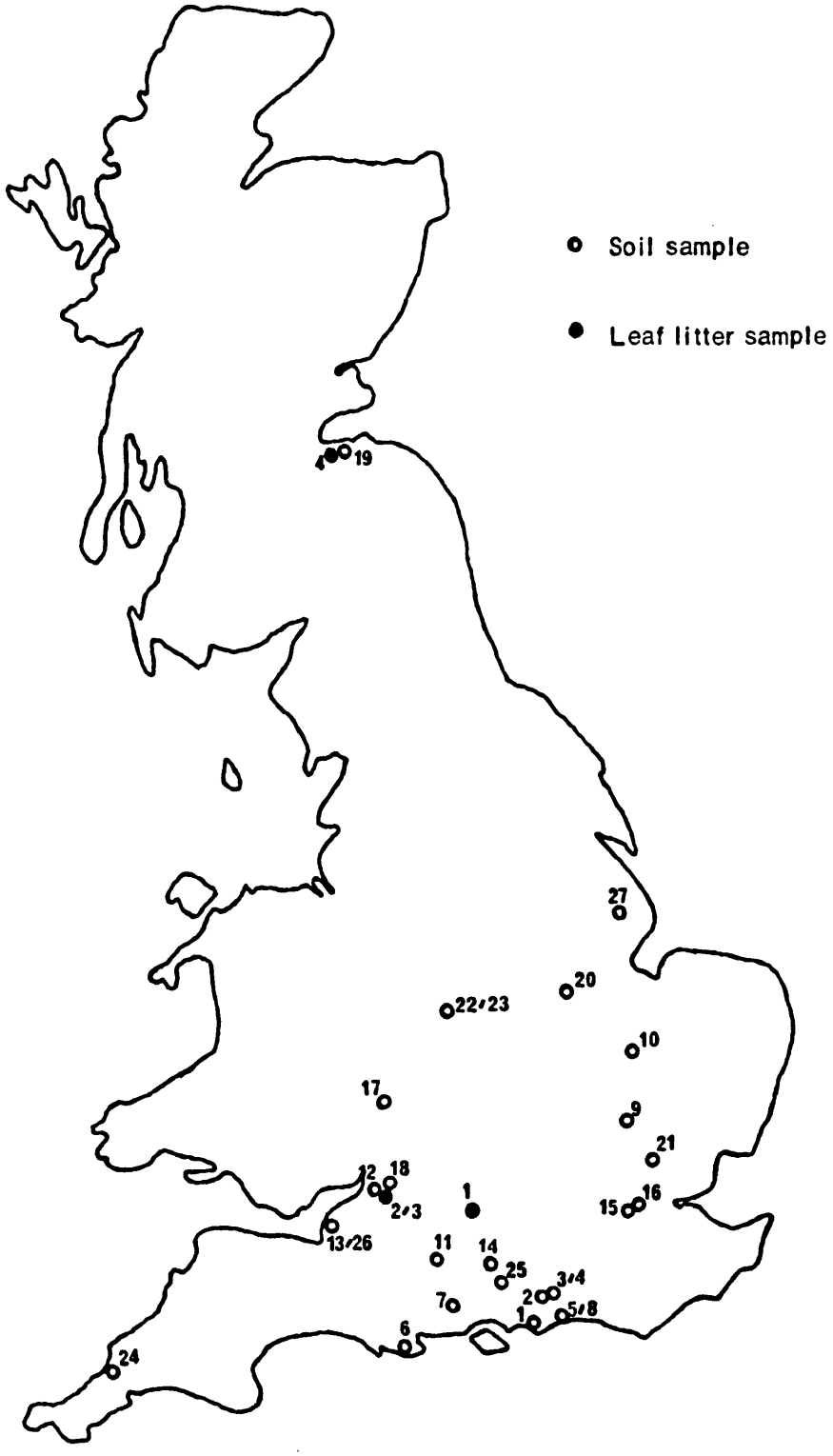


Fig.21. Soil and leaf litter sites other than at G.C.R.I.
The figures refer to the key on the following pages.

Key to soil sample sites. 1. GCRI sites.

1. Border close to the Institute's mushroom unit which was kept free from weeds and was backed by a 3 year old ornamental conifer hedge. pH approximately 4.5.

2. An established lawn. pH approximately 4.7.

3. A field under cultivation. During the sampling period the field was occupied with wheat stubble from a crop harvested the previous September (Jan-Apr) and with main crop potatoes (May-Nov). pH approximately 6.5.

4. A stand of mixed sweet chestnut and maple approximately 60 years old with an undergrowth of brambles and ivy. pH approximately 3,7. This site is referred to in the text as the deciduous wood.

5. A stand of Scots pine approximately 30 years old with a thick leaf litter carpet and little undergrowth. pH approximately 4.0. This site is referred to in the text as the coniferous wood.

Key to soil and leaf litter sample sites 2. Sites other than GCRI

a. Soil sites

Code	Date	Site	Soil type/cover	pH
1	Oct.79	Chichester W. Sussex	Deciduous wood Humus	3.9
2	"	Bedham W. Sussex	"	2.7
3	"	Lavington W. Sussex	"	3.2
4	"	"	Sandy heath Calluna	3.7
5	"	Rustington W. Sussex	Garden border Clay loam	7.3
6	Jan.80	Lulworth Dorset	Rough grass Sandy gravel	7.4
7	"	Ringwood Hants.	Rough grass Clay	7.3
8	"	Rustington W. Sussex	Garden border Clay loam	7.4
9	"	Harlow Essex	Rough grass Clay	7.7
10	"	Cambridge	Turf Dark loam	7.2
11	Feb.80	Over Wallop Hants.	Rough grass Clay loam	7.5
12	"	Frampton Cotterell Avon	Garden border Clay loam	7.6
13	"	Weston-S-Mare Avon	"	7.5
14	"	Andover Hants.	Deciduous wood Humus	7.8
15	Mar.80	Barnes Middx.	No cover Gravelly loam	7.1
16	"	Shepherds Bush London	Garden border Gravelly loam	6.8
17	Apr.80	Malvern Worcs.	Turf, gorse Humus	3.4

Code	Date	Site	Soil type/cover	pH
18	Apr.80	Horton Avon	Deciduous wood Humus	4.8
19	Aug.80	Edinburgh Midlothian	Garden border Loam	7.6
20	"	Peterborough Cambs.	Rough grass Clay	7.9
21	"	Theydon Bois Essex	No cover Sandy loam	7.3
22	Sep.80	Warwick	Deciduous wood Humus	3.4
23	"	"	Rough grass Sandy loam	4.5
24	"	Bodmin Moor Cornwall	Peat	3.6
25	"	Petersfield Hants.	Coniferous wood Sandy loam	3.1
26	Oct.80	Weston-S-Mare Avon	No cover Clay	7.7
27	Nov.80	Moulton Lincs.	No cover Silt	7.6

b. Leaf litter sites

1	Apr.80	Greenham Common Berks.	Mixed gorse/oak surface litter
2	"	Horton Avon	Grass, umbellifer surface litter
3	"	"	Lime, oak, ash Slightly decomposed
4	Aug.80	Edinburgh Midlothian	Pine surface litter

the samples were transferred to the laboratory in clean plastic bags. The samples were air dried at room temperature for 5 days, ground through a 2 mm sieve and quartered until approximately 10 g sub-samples remained. These were each accurately weighed to 10 g and shaken in 100 ml of sterile 0.1% water agar in stoppered 250 ml flasks for 10 minutes on a wrist action shaker. The resulting suspensions were diluted 100 fold with water agar, and 0.1 ml of diluted suspension was transferred to the surface of 4 replicate plates of freshly prepared selective medium. Water agar was used as the diluent because soil particles remain in suspension more readily in this medium than in water. The suspensions were spread over the agar surface with a flamed bent glass rod. This dilution rate gave 20 - 200 colonies per plate.

Further sub-samples of approximately 0.01 g were evenly distributed over the surface of 4 replicate plates of selective medium from the tip of a fine scalpel.

The pH of each soil sample was determined by suspending a 10 g sub-sample in 25 ml of 0.01M $CaCl_2$, mixing thoroughly and allowing the suspension to stand for 10 minutes. The pH was measured after a further brief mixing, using a Pye-Unicam model 291 pH meter.

All the isolation plates prepared from soil and other substrates were incubated in the dark for 3 weeks at 20° and were then examined using a binocular microscope. Colonies with verticillate branching were transferred to PDA plates and slopes for identification and storage.

Results

GCRI sites

Because of the selective nature of the medium used in the isolation studies, few conclusions could be drawn about the general fungal microflora of the different soil sites. The genera most frequently

occurring on the isolation plates were Penicillium, Fusarium, Cephalosporium and Verticillium. Both Fusarium and Cephalosporium were common in the cultivated field and turf sites whilst Penicillium spp. were predominant in the woodland sites.

The recovery of Verticillium spp. from the 5 GCRI sites throughout the sampling period is shown in Figs. 22 and 23, together with monthly rainfall and soil temperature at 10 cm. The results from each plating method are expressed both as the total number of Verticillium colony forming units (c.f.u.) recovered at each sampling and as the number of c.f.u. g^{-1} air dried sample. Because of the difference in amounts of soil applied to the isolation plates by the direct and dilution plating methods, 1 isolate recovered by the former method is equivalent to 25 c.f.u. g^{-1} and by the latter, 2500 c.f.u. g^{-1}. The total numbers of isolates recovered were 167 by direct and 114 by dilution plating.

Regular measurements of pH showed that there was little change at the different sites throughout the year.

At all sites, the majority of isolates were recovered in the summer and autumn months, although isolates from the two directly plated woodland sites were also relatively abundant in the spring. Using the direct plating method the recovery of isolates from woodland sites showed a marked periodicity throughout the year, but this was not so clearly marked in results from dilution plates prepared from the same samples. With both isolation methods however, the majority of the isolates were recovered from the woodland sites.

Despite the superficial similarity of the numbers of isolates recovered from the two directly plated woodland sites, the results correlated poorly (correlation coefficient = 0.53). This is probably due to the later peak in the numbers of isolates recovered from the

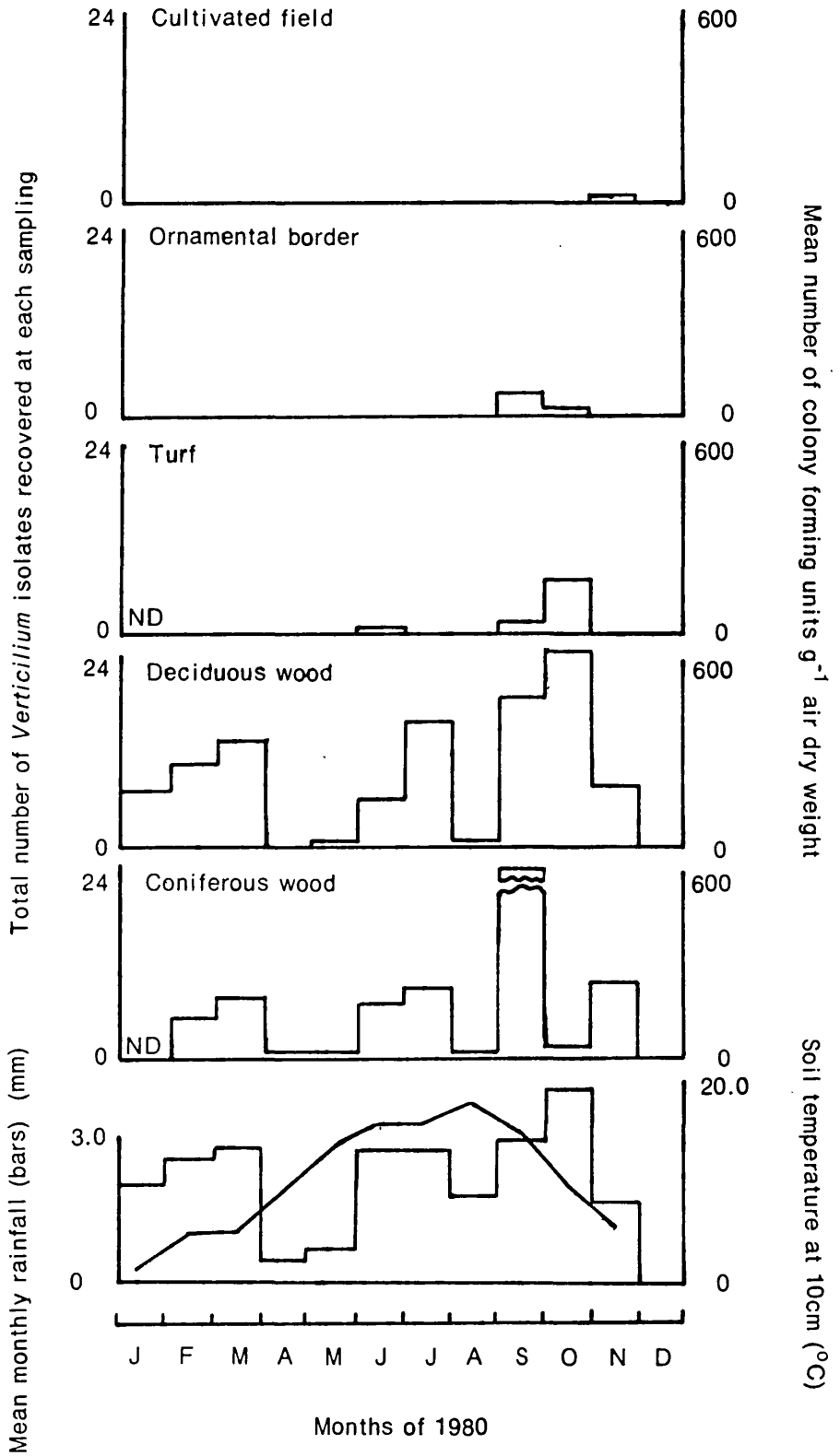


Fig.22. The recovery of *Verticillium* isolates from the 5 GCRI soil sample sites using the direct plating method.
ND: Not determined

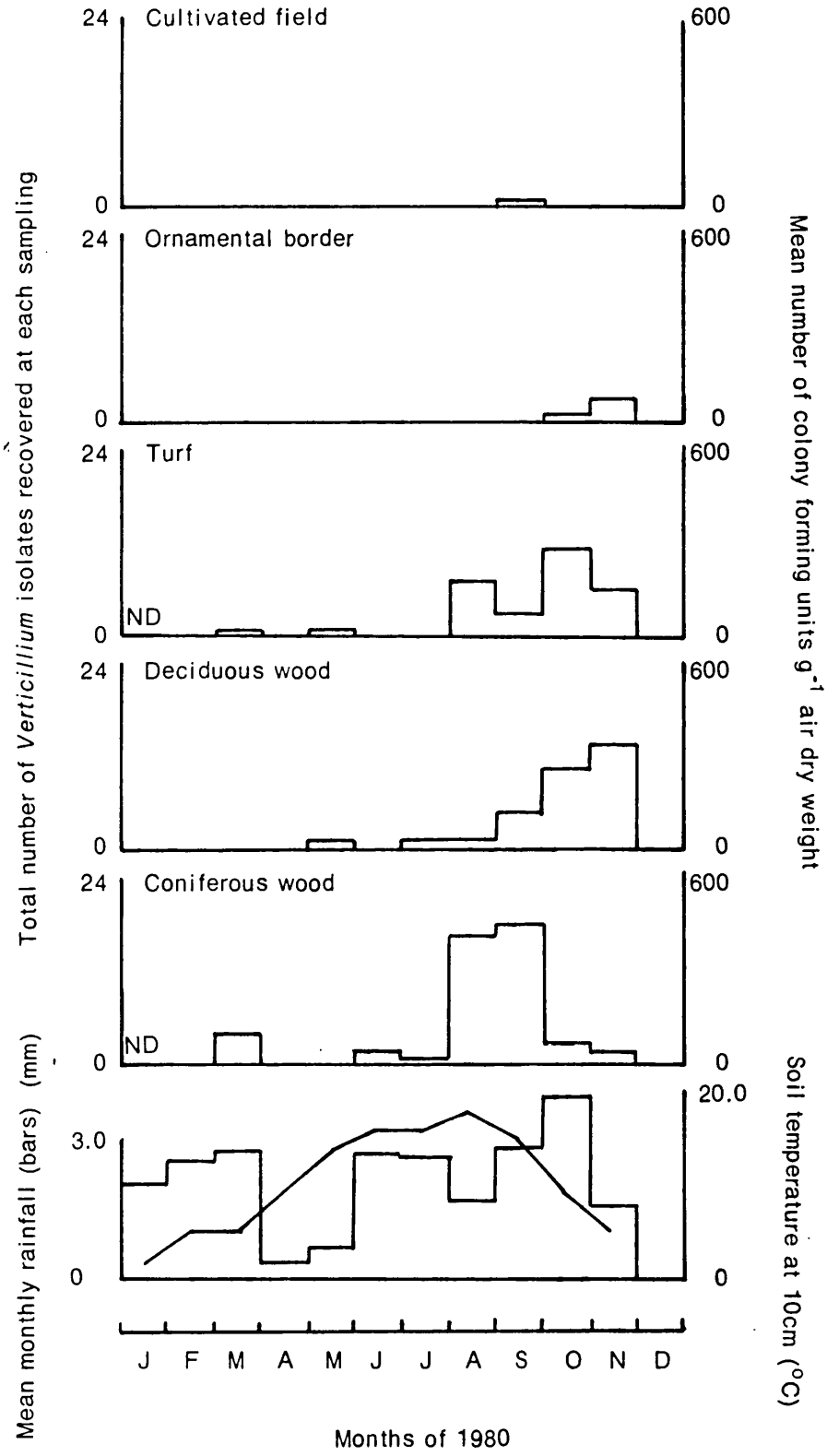


Fig.23. The recovery of *Verticillium* isolates from the 5 GCRI soil samples using the dilution plating method.
ND: Not determined.

deciduous wood site than from the coniferous wood site.

Because of the diverse nature of the sites studied, it is likely there were qualitative as well as quantitative differences in the species of Verticillium present at each site. However, the difficulties encountered in identifying the individual isolates meant that a detailed analysis of the relative abundance of different species could not be made.

Over 100 isolates were tested for pathogenicity to cut mushroom sporophores and 7 were included in the 'field' pathogenicity assay. All but one of these isolates were excluded from V.fungicola on the grounds of pathogenicity in the in vitro test. This isolate, obtained from the turf site in August 1980, achieved a score of 8 although it was morphologically atypical of pathogenic isolates of V.fungicola. None of the isolates tested in the 'field' pathogenicity assay produced symptoms comparable to dry bubble.

Isolates producing dictyochlamydospores (DCs) occurred at all of the sites except the cultivated field and formed a variable but often high proportion of the total number of isolates recovered, particularly in the summer and autumn samplings (Table 28). The two isolation techniques may have differed in their selectivity for DC producing isolates. For example, all of the isolates obtained from the border site by the dilution plating method produced DCs whilst none of those isolated by the direct plating method did so.

Sites other than the GCRI.

The geographical distribution and numbers of isolates obtained from the 27 non-GCRI sites are shown in Fig. 24. Although no Verticillium isolates were obtained from 16 of the sites, the 11 sites from which

Table 28. Percentage of the total number of isolates from the GCRI soil sites which produced dictyochlamydospores. The results for both plating methods are shown. The presence of dictyochlamydospores was determined after 7 days growth on PDA at 20 °C.

a. Direct plating of samples

Site	Month										
	J	F	M	A	M	J	J	A	S	O	N
Field	-	-	-	-	-	-	-	-	-	-	0
Border	-	-	-	-	-	-	-	-	0	0	-
Turf	ND	-	100	-	-	ND	-	0	100	84	0
Deciduous wood	50	50	14	-	100	ND	25	100	35	80	0
Coniferous wood	ND	80	25	100	100	ND	80	100	22	0	0

b. Dilution plating of samples

Site	Month										
	J	F	M	A	M	J	J	A	S	O	N
Field	-	-	-	-	-	-	-	-	0	-	-
Border	-	-	-	-	100	-	-	-	100	100	100
Turf	ND	-	-	-	0	ND	-	0	0	8	0
Deciduous wood	ND	-	-	-	0	ND	50	100	100	50	0
Coniferous Wood	ND	ND	0	-	-	ND	0	22	30	100	0

\- No isolates obtained

0 None of the isolates obtained produced dictyochlamydospores

ND Not determined

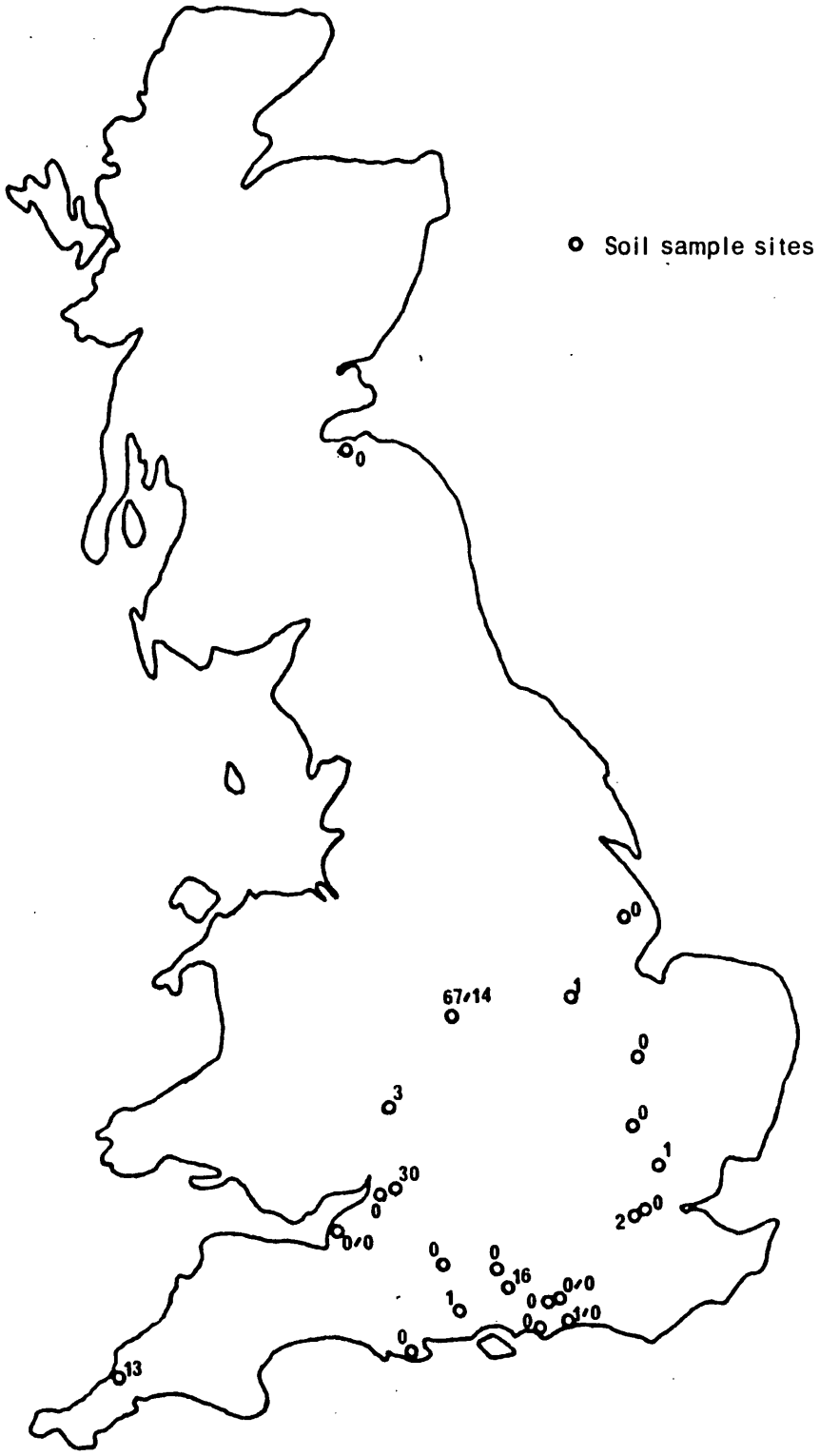


Fig.24. The distribution and frequency of isolates of *Verticillium* at soil sample sites around the U.K. The figures represent the total number of isolates recovered at each location using both isolation techniques.

they were recovered were of fairly widespread distribution. The frequency distribution of the numbers of isolates obtained at the different sites is summarised in Table 29. The numbers recovered varied considerably, over half of the sites yielding three or less isolates whilst one site (Warwick Castle) yielded over 60 isolates. The results of the two isolation techniques were broadly similar although, as with the GCRI sites, there were large quantitative differences between the methods when the results were expressed on a unit weight basis.

Evidence for a seasonal fluctuation in the numbers of isolates recovered is scant (Table 30) because of the irregular sampling dates, the different numbers and natures of the sites examined and the bias accorded by the high number of isolates obtained from the Warwick site.

Local topography is probably more important in determining the distribution of Verticillium species within the area surveyed than the geographical location. The sample sites were therefore classified as to soil type or vegetation cover and the distribution of isolates within each category is shown in Table 31. The majority of isolates were obtained from deciduous woodland sites where the soil generally had a high organic matter content and a low pH, although once again this result was considerably biased by the large number of isolates obtained at the Warwick Castle site. Isolates were also obtained from coniferous woodland soil, both sandy and clay loams and peat. Again, the direct and dilution plate isolation methods gave a similar pattern of results.

None of the 85 isolates included in the in vitro pathogenicity test or the single isolate included in the field pathogenicity test proved to be pathogenic and all were therefore excluded from V.fungicola.

Because of the difficulties in the identification of isolates there was little evidence of species variation between the sites. The distribution of DC forming isolates in the different soil types is

however shown in Table 32. Such isolates were notably absent from the clay loam type, which usually had a grass cover, but occurred in high proportions in both woodland soils and peat. As with the GCRI sites, there was a tendency for the dilution plating method to select DC forming isolates preferentially.

Table 29. The frequency of occurrence of isolates of Verticillium at the 27 sample sites around the U.K.

Number of isolates obtained from each site	Number of sites from which isolates were obtained	
	1. Direct plating	2. Dilution plating
0	18	19
1	3	2
2	1	1
3	0	0
4	0	0
5	0	0
6 - 15	4	3
16 - 25	0	2
26 - 35	0	0
above 35	1	0
	27	27

Table 30. The seasonal distribution of Verticillium isolates from the non-GCRI sampling sites.

	1979			1980									
	O	N	D	J	F	M	A	M	J	J	A	S	O	N
Number of isolates obtained	Direct plating													
	1	-	-	1	0	2	9	-	-	-	0	63	0	0
	Dilution plating													
	0	-	-	0	0	0	24	-	-	-	2	47	0	0
Number of sites from which isolates were obtained	1	-	-	5	4	2	2	-	-	-	3	4	1	1

Table 31. Distribution of Verticillium isolates between the 7 soil types examined.

Soil type	Number of isolates obtained by Direct plating	Dilution plating	No. of sites
Deciduous wood	50	47	2
Coniferous wood	7	9	1
Loam-clay loam	5	1	3
Sand-sandy loam	6	8	2
Gravel	0	0	2
Silt	0	0	1
Peat	8	8	2
	76	73	13

Table 32. The percentage of Verticillium isolates at each site within the 7 soil types examined that produced dictyochlamydospores after 7 days growth on PDA at 20°.

	Direct Plating			Dilution Plating		
	Site No.			Site No.		
	1	2	3	1	2	3
Soil type						
Deciduous wood	13	79	-	82	92	-
Coniferous wood	86	-	-	89	-	-
Loam-clay loam	0	0	0	0	0	0
Sand-sandy loam	71	0	-	29	100	-
Gravel	0	0	-	0	0	-
Silt	0	-	-	0	0	-
Peat	100	100	-	50	50	-

\- No isolates recovered

0 No DC forming isolates recovered

Discussion

GCRI sites

Verticillium species had a widespread distribution in the sites examined at the GCRI, the highest numbers of isolates occurring in the woodland soils and the lowest in the border and cultivated field soils. There was also evidence of a seasonal variation reflected in the number of isolates recovered. Previous studies on seasonal variations in the soil microflora that have included *Verticillium* species have suggested that populations tend to a maximum in spring and summer (Bisset & Parkinson, 1980; coniferous woodland soil; Widden & Parkinson, 1973; coniferous woodland soil; Witkamp, 1960; deciduous woodland soil). Gams and Domsch (1969) found that of 12 fungi studied in cultivated soils, only *V.nigrescens* had a statistically significant seasonal fluctuation, again with a maximum population in the summer months. The findings from this study indicate, however, that at all sites investigated the population of *Verticillium* species was maximal in the late summer and autumn.

The periodicity of the seasonal distribution (Figs. 22 & 23), which was especially marked in the directly plated deciduous woodland samples, was shown by the paucity of isolates recovered in April and May and again in August. The first was a period of very limited rainfall and the poor recovery of isolates may have been due to a reduction in the viability of the population under conditions of moisture stress. Rainfall was also limited in August although to a lesser extent, but again reduced water availability possibly coupled here with the high soil temperature may have been a factor in the low recovery of isolates.

There was no parallel reduction in recovery of isolates from the dilution plated coniferous wood sample in August although conversely, the recovery of isolates from the woodland sites in July was much less

using this method than the direct plating method. The reasons for these discrepancies between the methods are not clear although Sewell (1959) has underlined the great variability encountered in attempts to identify seasonal fluctuations in numbers of soil fungi and also indicated that whilst one isolation method may sucessfully identify a seasonal variation, a second may not and this possibly applies here.

Sewell also suggested that different methods of investigation may give different qualitative and quantitative views of the composition of the soil microflora. Although the two methods employed here gave a broadly similar picture of the seasonal pattern of distribution of isolates there were large differences in the estimates of the numbers of isolates recovered per unit weight of sample between the two methods. Whilst the dilution plate method may underestimate the fungal population as discussed in the previous section, the direct method of isolation gave an even lower estimate. This may in part be due to a low degree of contact between the directly plated sample and the agar surface and consequently inoculum may have been trapped within the soil particles. Individual crumbs usually gave rise to a single colony of Verticillium although such relatively large particles are likely to have contained more than one c.f.u. In the dilution method, the soil crumbs are more thoroughly dispersed and a greater amount of inoculum may be released. Other sources of error in the two methods may arise during the preparation of the dilution series and because of the difficulties in ensuring that comparable amounts of inoculum were used when samples were directly plated.

Sites other than GCRI

Non-vascular plant pathogenic and saprophytic Verticillia have been isolated from a wide range of soil types of widespread geographical distrubution but because of taxonomic difficulties they have rarely been identified as to species. Sites from which such Verticillium species

have been isolated include coniferous woodlands in Sweden (Bååth & Soderstrom, 1980; Soderstrom, 1975), Canada (Bisset & Parkinson, 1980; Widden & Parkinson, 1973) and the U.K. (Ellis, 1940; Hayes, 1965); heathland sands in the U.K. (Jefferys et al., 1953; Thornton, 1956); fenland peat in the U.K. (Stenton, 1953); estuarine salt marshes in the U.K. (Elliott, 1930) and pasture soils in New Zealand (Jackson, 1965). Both V.fungicola and V.psalliotae have been infrequently isolated from soil but none of the isolates tested in this study proved to be pathogenic.

Verticillium isolates were most common in the woodland sites which confirms the GCRI site data and that of other investigators who have mainly concentrated on coniferous woodland soils. The results of this study suggest that Verticillium species may also be common components of the microflora of deciduous woodland soils. DC forming isolates were common in both woodland soil types.

The incidence of Verticillium species in native peats is particularly interesting in view of the use of this substrate as a constituent of the casing layer.

b. Leaf litter

As well as occurring commonly in soils, Verticillium species have also been recorded in various types of leaf litter (Gams, 1971; Kjoller & Struwe, 1980) and the report of V.fungicola from this substrate (Brady & Gibson, 1976) prompted an investigation of the woodland leaf litters at the GCRI and at 3 other sites in the U.K.

Materials and Methods

Because of the broadly similar picture of the distribution of Verticillium species in soil given by the two plating methods and the greater convenience of direct plating, this method alone was used in the

investigation of other substrates.

Approximately 15 g samples of leaf litter were taken at three stages of decomposition in the two woodland stands at the GCRI in March, May and October 1980. The stages of decomposition were:-

1. Surface litter that consisted mainly of dry whole leaves or needles = the L layer;

2. Subsurface litter that consisted of darkened, partially decomposed plant remains = the F layer;

3. Well humified litter taken from above the soil surface in which the individual components were not clearly recognisable = the H layer.

Two further litter samples were taken at the GCRI, one consisting of decomposing wheat straw collected from stubble collected in March 1980 and the second consisting of fresh grass clippings collected in July 1980. Samples collected at other sites around the U.K. are described in the Key to Figure 21 above.

Care was taken not to cross contaminate the different layers and all samplings were made with alcohol sterilised implements. Samples were transferred to the laboratory in clean plastic bags, air dried at room temperature and were milled to a fine dust. Approximately 0.005 g sub-samples were sprinkled over the surface of 4 replicate plates of selective medium per sample and the plates were incubated and examined as described above.

Results

The occurrence of isolates of *Verticillium* in the GCRI site litter samples and their distribution in the litter profile is shown in Fig.25. The total number of isolates obtained from the two sites was similar, 40 from the deciduous and 38 from the coniferous wood. The numbers of isolates per unit weight were greater than in the directly plated soil

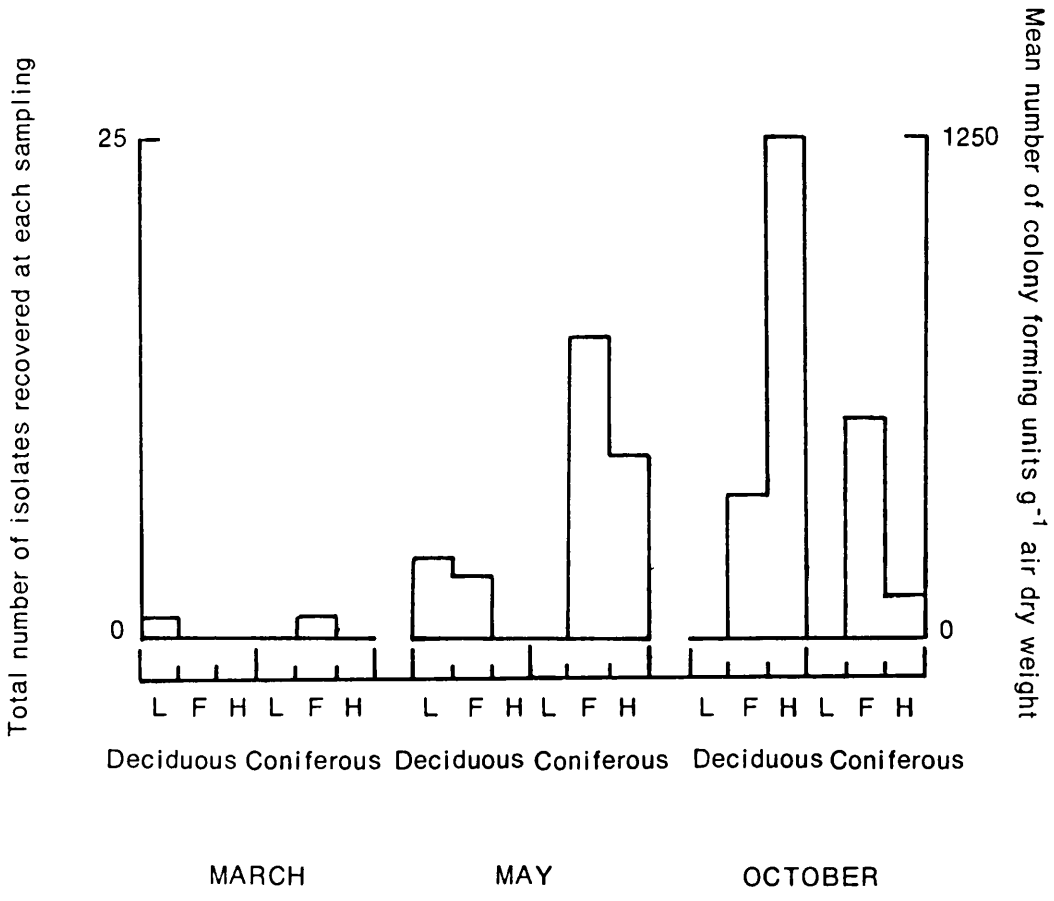


Fig.25. The recovery of *Verticillium* isolates from 2 woodland leaf litters at the GCRI during 1980.
L : dry surface litter
F : sub-surface litter , some decomposition and humification
H : litter directly above soil surface, well humified

samples.

The greatest total number of isolates were recovered in the October sampling at the deciduous wood site and in the May sampling at the coniferous site. At the latter site, the majority of isolates at each sampling came from the F horizon and no isolates were obtained from the dry surface litter. By contrast, isolates were obtained from the surface litter in the March and May samplings at the deciduous wood site. In the October sampling they were found further down the profile with maximum numbers recovered in the H layer.

Isolates producing DCs were not found in March but occurred with similar frequencies at both sites in the May and October samplings (Table 33). Isolates tentatively identified as V.lecanii occurred only in the L layer of the deciduous wood litter in the March and May samplings but occurred only in the lower litter horizons in the October sampling (Table 34).

One isolate of Verticillium was recovered from the straw litter collected at the GCRI and this was identified as V.lamellicola by W.Gams.

Verticillium spp. were isolated from two of the three sites sampled around the U.K. and the deciduous litter collected at Horton, Avon, yielded several isolates morphologically similar to V.lecanii. No Verticillium isolates were recovered from the Pinus surface litter collected in Edinburgh which confirms the observations made at the GCRI coniferous woodland site.

Fifty-one isolates were included in the in vitro pathogenicity test, one of which (from deciduous wood surface litter, Horton, Avon) attained a score of 8 and was thus comparable to V.fungicola. As with the high scoring isolate recovered from the GCRI site, this isolate was not morphologically typical of V.fungicola isolated from mushrooms. Seven isolates were included in the field pathogenicity assay but none produced symptoms comparable to V.fungicola.

Table 33. Percentage of isolates from leaf litter that produced dictyochlamydospores after 7 days incubation on PDA at 20°.

	March		May		October	
	DW	CW	DW	CW	DW	CW
Horizon						
L	0	-	0	-	-	-
F	-	0	100	100	55	50
H	-	-	ND	33	25	50

DW = deciduous wood site

CW = coniferous wood site

Table 34. Percentage of isolates from leaf litter that were tentatively identified as V.lecanii.

	March		May		October	
	DW	CW	DW	CW	DW	CW
Horizon						
L	100	-	100	-	-	-
F	-	0	0	0	15	0
H	-	-	ND	0	4	0

DW = deciduous wood site

CW = coniferous wood site

Discussion

The results suggest that Verticillium species are common inhabitants of both coniferous and deciduous woodland leaf litters and indicate that there are marked seasonal changes in abundance in the different litter horizons.

Apart from isolates producing DCs and those that were included within V.lecanii, none of the isolates in the study were satisfactorily identified. However, reports of Verticillium species obtained from leaf litter include V.terrestre, V.candelabrum, V.lateritium, V.cellulosae, V.lecanii, V.bulbillosum, V.psalliotae and V.fungicola. (Bååth & Soderstrom, 1980; Bisset & Parkinson, 1980; Brady & Gibson, 1976; Brandesburg, 1969; Soderstrom, 1975)

Variation in the frequency of Verticillium species occurring at different depths in the litter horizon has also been noted in other studies. Soderstrom (1975) reported that the DC forming species V.bulbillosum was most frequently associated with the F and H layers in a coniferous woodland litter and the findings for the DC forming isolates in this study tended to confirm this.

Seasonal variations in the frequency of Verticillium species in leaf litter have also been previously noted. For example, Bissett and Parkinson (1980) observed a high frequency of V.lecanii in coniferous woodland litter which reached a maximum during the summer months. Although no isolates of V.lecanii were obtained from the GCRI coniferous wood in this study, this species of Verticillium alone was recovered in the March and May samplings in the deciduous wood and may be an important constituent of the litter microflora. Kjoller and Struwe (1980) observed that an unidentified Verticillium species occasionally formed an important component of the microflora of red alder litter, although on this substrate the maximum occurrence was in the spring.

A single isolate from litter could be included within V.fungicola on the basis of in vitro pathogenicity, but as with the high scoring soil isolate, it was not morphologically typical of V.fungicola isolated from mushrooms. This isolate was not included in the 'field' pathogenicity test and therefore its ability to reproduce the entire disease syndrome remains uncertain.

The role of Verticillium species within both leaf litter and soil is not clear although it seems likely that different species may occupy different ecological niches. Bååth and Soderstrom (1980) attempted to explain the presence of a range of fungi in different soil horizons in terms of their ability to degrade macromolecules likely to be present (eg. protein, chitin, cellulose and xylan) but found few consistent trends. However, V.bulbillosum isolated from conifer litter degraded protein, chitin and xylan but was poorly cellulolytic, whilst V.lecanii degraded protein and chitin alone. The ability to degrade these substrates suggests that these fungi may be involved in their breakdown in both soil and litter and the degradation of chitin in particular correlates with the ability of species such as V.lecanii to attack chitin rich substrates such as insects and other fungi.

c. Casing materials

The application of non-sterilised peat to mushroom beds as a constituent of the casing prompted a previous investigation of its possible role as a source of inoculum of V.fungicola but with little success (L.Jacobs, pers com.). Species of Verticillium have been shown to occur in natural peat (Dickinson & Dooley, 1967) and the failure to isolate them from commercial samples may have partially been due to unsatisfactory isolation techniques.

Little is known of the microbiology of crushed chalk which is

commonly used to neutralise acidity in peat based casing.

Both of these substrates were examined for the presence of Verticillium spp. by direct plating onto the selective medium.

Materials and Methods

1. Peat

Using an alcohol sterilised spatula, approximately 5 g samples were taken from 43 freshly opened 50 kg bales of 'Irish moss' Sphagnum peat. The samples were transferred to the laboratory in clean plastic bags and approximately 0.005 g (fresh weight) sub-samples were sprinkled over the surface of 4 replicate plates of selective medium per sample. The plates were incubated and examined as described previously.

2. Chalk

Approximately 30 g samples were taken from 9 freshly opened bags of 'Duncton' crushed chalk and 0.01 g samples of the finely divided dust were sprinkled onto 4 replicate plates of selective medium per sample. The plates were incubated and examined as described previously.

Results

1. Peat

Of the 43 bales of peat sampled, 34 yielded a total number of 86 isolates of Verticillium. The number of isolates obtained per sample ranged from 0 - 6 giving an approximate concentration of from 0 - 300 cfu g^{-1} fresh weight (Table 35).

In an attempt to identify the isolates to species level, 57 isolates were morphologically examined but with inconclusive results. Seventeen isolates were also sent to Dr W Gams at the CBS who assigned them to 4 species: V.lecanii, V.lamellicola, V.psalliotae and V.bulbillosum.

The proportion of the total number is isolates which I have tentatively assigned to these species is shown in Table 36.

Table 35. Frequency distribution of the number of isolates of *Verticillium* in peat bales.

No of isolates per sample	Approx. No. cfu g^{-1} fresh weight	No. of bales within each frequency class
0	0	9
1	50	13
2	100	7
3	150	5
4	200	6
5	250	3
6	300	1

Table 36. The proportion of the total number of isolates tentatively assigned to 3 species of *Verticillium*. DC producing isolates are *V.bulbillosum*, *V.chlamydosporium* and *V.catenulatum*.

Species	% of total numbers of isolates
V.lecanii	19
V.psalliotae	6
V.lamellicola	1
DC producing isolates	35
Rest	39

Seventy isolates were included in the in vitro pathogenicity assay and 30 in the 'field' assay. None of the isolates produced symptoms comparable to V.fungicola although in the field assay some cap spotting may have been attributable to certain isolates (see page 94).

2. Chalk

The fungal microflora of crushed chalk as revealed by colonies developing on the selective medium was limited both in size and diversity. Three isolates of Verticillium were obtained from the 9 bags sampled, the majority of other fungi isolated being Penicillium spp. Two of the Verticillium isolates (CH1 and CH3) occurred frequently in their respective samples. Isolate CH2, which was of more limited occurrence but which was morphologically similar to CH3, was identified as V.leptobactrum by W Gams.

Isolates CH2 and CH3 failed to score in the in vitro pathogenicity assay. Isolate CH1 however scored a maximum of 10 although it grew slowly and sporulated poorly on PDA, which are characters atypical of V.fungicola. None of the isolates were included in the 'field' pathogenicity assay.

Discussion

The results demonstrate that Verticillium species, including two commonly associated with mushroom cultivation (V.psalliotae and V.lamellicola), are of frequent occurrence in horticultural grade Sphagnum peat. Although many were morphologically identical to V.fungicola, none of the isolates were pathogenic to A.bisporus.

Verticillium species were also isolated from samples of natural peat during the present study and several reports have suggested that they are common components of the microflora of moorland peat. In an

investigation of the microbial colonisation of disused peat workings, Dickinson and Dooley (1967) observed that Verticillium species were, along with Trichoderma viride and Morteriella species, the most common fungi isolated from the surface layers of drained, raised acid bogs which supported a mixed vegetation of Calluna, Erica and Eriophorum. Christensen and Whittingham (1965) also isolated Verticillium from Sphagnum bogs in Wisconsin and Singh (1980) recorded Verticillium species from the rhizospheres of the typical wet heathland plants Oxycoccus palustris and Deschampsia flexuosa. Verticillium species are not restricted to acid peats; Stenton (1953) frequently isolated Verticillium from the surface layers of fen peats in Cambridgeshire. None of the isolates were identified to species level in these studies.

As with the other substrates examined, the role of Verticillium species in the ecology of peat is unknown.

The widespread occurrence of Verticillium species in peat may have important implications as regards the use of unsterilised peat as a component of the casing. Although V.fungicola was not identified in any of the samples examined, it may be unwise to disregard the possibility of mushroom pathogenic isolates being present in peat. Certain isolates (see page 94) which are morphologically very similar to V.fungicola may be able to cause spotting of pilei under field conditions although they failed to reproduce the entire desease syndrome. Two other species of Verticillium which under certain conditions can develop on cultivated mushrooms were also isolated from peat. These were V.lamellicola, which frequently develops on A.bisporus sporophores that are stored under humid conditions at room temperature, and V.psalliotae which attacks A.bitorquis at the higher temperatures required for the cultivation of this fungus. A recent attempt by a Surrey grower to produce a commercial crop of A.bitorquis resulted in a pileus spotting disease caused by V.psalliotae which had not been previously recorded on the farm.

(Upstone & Carter, 1979). It seems likely that the casing peat was the source of inoculum for this outbreak of disease.

If pathogenic strains of Verticillium are present in peat it may seem surprising that primary outbreaks of dry bubble attributable to contaminated peat are not of more widespread occurrence. The results indicate however that the amount of Verticillium present per unit weight was fairly low even when allowance was made for the inefficiency of the direct plating method in the recovery of inoculum. Successful artificially induced outbreaks of dry bubble require relatively large inocula (Holmes, 1971) and disease severity also appears to be linked to inoculum concentration (Gandy, 1970). It is therefore possible that there is generally an insufficient concentration of inoculum in peat to incite disease (low inoculum potential) and primary outbreaks may be caused by chance concentrations of inoculum coinciding with favourable environmental factors.

Steaming has been advocated as a method of eliminating V.fungicola in casing suspected of contamination (Gandy, 1970; Wuest et al, 1970; Wuest & Moore, 1972). Whilst this may be a useful practice under certain conditions, the routine steaming of casing on farms where dry bubble is endemic may only serve to exacerbate the disease by allowing the pathogen to 'luxuriate' in the pasteurised substrate (Baker & Cook, 1974; Treschow, 1941).

The fungal microflora associated with the crushed chalk was limited, but the survival of any fungi in such a seemingly inhospitable environment was surprising. Although no counts were made, a fairly high proportion of the colonies recovered on the selective medium were Verticillium species. This is particularly interesting in view of the findings of Went (1969) who frequently and consistently isolated V.lamellicola (as Cephalosporium lamellicola FEV Smith) from the tips of developing stalactites in limestone caves. Went implicated this fungus in stalactite

formation through the stimulation of deposition of calcium carbonate by the action of the enzyme carbonic anhydrase. However, Hasselbring et al (1975) contested the unique role of V.lamellicola in stalactite formation by isolating other fungi (Penicillium cyclopium, Fusarium moniliforme and Mucor spp.) from stalactite tips. These authors do not state whether V.lamellicola was also isolated in their study. Interestingly, V.lamellicola is morphologically similar to V.leptobactrum (isolate CH2).

The significance of the high score of isolate CH1 in the in vitro pathogenicity test is uncertain as the isolate was not included in the field pathogenicity test because of its poor sporulation.

V.lamellicola is not the only microorganism associated with the cultivated mushroom to be isolated from chalk. In a recent survey of possible sources of inoculum of the bacterial blotch disease of mushrooms, Wong and Preece (1980) found casing chalk to be contaminated with the causal bacterium Pseudomonas tolaasi (Paine).

Further taxonomic research, coupled with extensive sampling, would help to clarify the possible role of peat and chalk in the epidemiology of mushroom diseases caused by Verticillium species.

d. Fungi

A summary of reported associations between Verticillium species and other fungi is presented in Table 37. Many of the 'host' fungi are either Basidiomycetes with large, fleshy fruit bodies or plant pathogenic micro-fungi and associations between several species of Verticillium (including V.fungicola, V.psalliotae and V.lamellicola) and rusts are particularly common. The associations are not restricted to Basidiomycetes and the taxonomic spectrum of the 'host' species is fairly broad.

In relatively few cases is parasitism of the 'host' clearly demonstrated apart from those species causing diseases of the cultivated

Table 37. A summary of associations between *Verticillium* species and other fungi.

Species	Host	Reference	Disease
V.agaricinum Corda. = *V.lactarii*	*Russula* sp.	Wakefield & Bisby (1941)	NS
	Lactarius sp.	Isolated	
V.albo-atrum (R&B)	*Rhopalomyces elegans*	Barron & Fletcher (1970) Inoculated	+
V.arenarium (Petch) W Gams	*Puccinia graminis*	Pon et al (1959) Isolated	NS
V.cercosporae (Petr.)	*Cercospora hibisci*	Petrak & Ciferri Isolated	NS
V.dahliae Kleb.	*Rhopalomyces elegans*	Barron & Fletcher (1970) Inoculated	+
V.epimyces Berk. & Br.	*Elaphomyces* sp.	Wakefield & Bisby (1941) Isolated	NS
V.epiphytum Hansf.	*Helminthosporium triumfettae*;	Hansford (1943) Isolated	NS
	Hemileia vastatrix	Hansford (1943) Isolated	NS
V.fungicola	*A.bisporus*	Ware (1933) Isolated & inoculated	+
	A.bitorquis	Gandy unpublished Inoculated	+
	Laccaria laccata	C.B.S. Isolated	NS
	Coltricia perennis	Gams (1971)	NS
	Unidentified leaf fungi	Isolated	NS
	Unidentified woodland toadstool	Preuss (1851) Isolated	NS
	Rhopalomyces elegans	Barron & Fletcher (1970) Inoculated	+
	'Cereal rust'	Hassebrauk (1936) Isolated	+

Table 37 - continued.

Species	Host	Reference	Disease
V.fusisporum W Gams	Coltricia perennis	C.B.S. Isolated	NS
V.insectorum (Petch) W Gams	Tremella mesenterica	C.B.S. Isolated	NS
	Oidium aureum		
V.lamellicola (FEV Smith) W Gams	A.bisporus	Smith (1924) Isolated	+
	Puccinia arenaria	C.M.I. Isolated	NS
	Polyporus varius	C.B.S. Isolated	NS
	Hypoxylon deustum	C.B.S. Isolated	NS
	Fomes fomentarius	C.B.S. Isolated	NS
V.lateritium Rabenhorst	Alternaria brassicae	Tsuneda et al (1976) Isolated & inoculated	+
V.lecanii (Zimm.) Viegas	Puccinia chrysanthemi	Kotthoff (1937) Isolated as V.coccorum	NS
	Puccinia coronata	C.B.S. Isolated	NS
	Puccinia graminis	McKenzie & Hudson (1976)	+
	Puccinia dianthae	Spencer (1980) Inoculated	+
	Hemileia vastatrix	Bouriquet (1947) Isolated as V.hemileiae	+
	Hemileia scholtzii	C.B.S. Isolated	NS
	Uromyces laevigatus	C.B.S. Isolated	NS
	Uromyces appendiculatus	Hull, Allen & Leeming (in preparation)	+
	Cronatium asclepideum	Castellani & Graniti (1949) Isolated as V.coccorum	+

Table 37 - continued.

Species	Host	Reference	Disease
V.lecanii cont.	Unidentified rust on Erica	Sartory, Sartory & Meyer (1931) Isolated	NS
	Helvella lacunosa	C.B.S. Isolated	NS
	Tuber maculatum	C.B.S. Isolated	NS
	Erisyphe graminis	Hall, Allen & Leeming (in preparation)	+
V.leptobactrum W Gams	Lactarius rufus	C.B.S. Isolated	NS
	Merulius tremellosus	C.B.S. Isolated	NS
V.psalliotae Treschow	A.bisporus	Treschow (1941) Isolated	+
	A.bitorquis	Upstone & Carter (1975)	+
	Rhopalomyces elegans	Dayal & Barron (1970) Isolated & inoculated	+
	Absidia spinosa	Dayal & Barron (1970) Inoculated	+
	Cunninghamella echinulata	Dayal & Barron (1970) Inoculated	+
	Rhizopus stolonifer	Dayal & Barron (1970) Inoculated	+
	Thamnidium elegans	Dayal & Barron (1970) Inoculated	+
	Zygorhynchus moelleri	Dayal & Barron (1970) Inoculated	+
	Morteriella sp.	Dayal & Barron (1970) Inoculated	+
	Olivea colebrookeae	Sukapure & Thirumalacher (1966) Isolated	NS
	Puccinia coronata	C.B.S. Isolated	NS
	Hemileia vastatrix	C.B.S. Isolated	NS
	Puccinia sp.	C.M.I. Isolated	NS

Table 37 - continued.

Species	Host	Reference	Disease
V.psalliotae cont.	Uredo sp.	C.M.I. Isolated	NS
	Endogone lactiflua	C.B.S. Isolated	NS
	Tuber spp.	C.B.S. Isolated	NS
V.quaternellum Grove	Mycena sp.	Wakefield & Bisby (1941) Isolated	NS
V.rexianum (Sacc) Sacc.	Dictydium sp.	C.B.S. Isolated	NS
	Stemonitis fusca	C.B.S. Isolated	NS
	Trichia varia	C.B.S. Isolated (= V.niveostratum)	NS
Verticillium sp.	Unidentified toadstool	Gams (1971) Con. stat. of Cordyceps militaris (L) Link	NS

Isolated	Indicates that the Verticillium species was isolated from the naturally infected host species.
Inoculated	Indicates that the host species was artifically inoculated with and supported growth of the Verticillium species.
+	Recorded as causing disease
NS	Not stated
C.B.S.	Culture lists of Centraalbureau voor Schimmelcultures
C.M.I.	Culture lists of Commonwealth Mycological Institute

mushroom and most of the records are of single isolations. However, V.lecanii appears to be a destructive mycoparasite of several rust species (Garcia-Acha et al, 1965; Spencer, 1980) whilst the parasitism of Rhopalomyces elegans and other Zygomycetes by V.psalliotae appears to be more subtle (Dayal & Barron, 1970).

Because of the prevalence of associations between Verticillium species and fleshy Basidiomycetes and plant pathogenic microfungi, these sources were investigated as possible sources of inoculum of V.fungicola.

Materials and Methods

Collections of diseased Basidiomycete fruitbodies were made during the autumn of 1978 and the spring of 1980. The sporophores were incubated in humid chambers at room temperature and were periodically examined for the development of verticillate fungi or other symptoms characteristic of infection by V.fungicola. None of the specimens were satisfactorily identified but the majority were boletes.

Sporophores of 27 species in 21 genera of the Agaricales and one species in Gasteromycetales were inoculated with a concentrated suspension of V.fungicola conidia (c.10^6 ml^{-1}) and were incubated in humid chambers at room temperature. The sporophores were periodically examined for signs of infection.

A collection of 17 rust and powdery mildew fungi was made during the summer of 1980. Infected plant tissues bearing sporulating pustules or mycelium were incubated as described for the Basidiomycete sporophores and microscopically examined for the development of verticillate mycelium.

Nine species of rusts and powdery mildew fungi were also inoculated with a concentrated suspension of V.fungicola conidia, and were incubated and examined for development of V.fungicola mycelium and symptoms of parasitism.

Further studies were made on the ability of V.fungicola to attack cucumber powdery mildew (Sphaerotheca fuliginea (Schlecht ex Fr.). Discs 1 cm in diameter were cut from leaves of both healthy and mildewed 3 week old cucumber plants (cv. Long Green Ridge) and were floated adaxial surface uppermost on tap water in plastic cups (Spencer, 1980). The leaf discs were sprayed with either a washed suspension of V.fungicola conidia (c. 10^6 ml^{-1}) or with sterile distilled water and the cups were covered with Petri dish lids to maintain a high relative humidity. After 4 days incubation at room temperature the discs were cleared by floating on chloral hydrate, stained with cotton blue in lactophenol and the development of V.fungicola on discs with and without mildew was compared.

Other mildew infected discs on which V.fungicola had developed were tapped over plates of selective medium to determine whether V.fungicola could be dispersed in association with the mildew conidia. Uninoculated leaf discs and discs of agar cultures of V.fungicola were similarly treated and acted as controls. The plates were incubated at 20° and were periodically examined for the development of Verticillium colonies.

An attempt was made to determine if a nutritional relationship existed between the powdery mildew and V.fungicola. Mildew conidia were washed from cucumber leaves with a jet of distilled water and concentrated by centrifugation. The conidia were spread onto sterile glass slides in humid chambers and sprayed either with a washed suspension of V.fungicola conidia or with sterile distilled water and were incubated at 20°. The slides were periodically examined for associations between V.fungicola germ tubes and the mildew conidia.

A leachate of mildew conidia was also tested for the ability to stimulate the germination of washed V.fungicola conidia. Approximately 5 ml of a concentrated suspension of mildew conidia in sterile distilled water was shaken for 24 hours, centrifuged and the supernatant sterilised

by membrane filtration. An equal volume of sterile distilled water that had been run over the surface of healthy cucumber leaves was treated similarly. Drops (50 µl) of the filtrates were incubated with 20 µl drops of a washed suspension of V.fungicola conidia on cavity slides in humid chambers. The percentage germination of the V.fungicola conidia was measured after 24 hours incubation at 20°; the 2 treatments were compared using Student's t test.

The development of V.fungicola on whole mildew-infected cucumber plants was also studied. Two 5 week old cucumber plants bearing large colonies of mildew on the lower leaves were placed in a large Perspex box, the base of which was lined with water-soaked capillary matting to maintain a high relative humidity. The plants were sprayed with a washed suspension of V.fungicola conidia (c. 3×10^7 ml^{-1}), and were periodically examined for the development and spread of V.fungicola mycelium over the mildew colonies.

Results

No Verticillium species were isolated from the diseased Basidiomycete sporophores. Fungi isolated were predominantly Penicillium spp. although Sepedonium spp. were common on boletes. Many of the specimens were decomposed by bacteria before fungi developed.

The results of the inoculations of Basidiomycete fruit bodies with V.fungicola are shown in Table 38. Within the Agaricales the reaction to inoculation varied from no response (9 species in 8 genera) to a browning and sinking of tissue similar to that produced on A.bisporus (2 species in 1 genus). Intermediate responses included browning and tissue collapse below the infection drop in the absence of mycelium (1 species) and the development of surface mycelium in the absence of tissue collapse (10 species in 8 genera).

Table 38. The effect of inoculating Basidiomycete sporophores with conidial suspensions of V.fungicola.

Species	Reaction to inoculation
Basidiomycetes	
a. Agaricales	
Agaricus arvensis	D
A.macrosporus	C
A.silvicola	D
Agrocybe sp.	C
Amanita citrina	C
Clitocybe nebularis	C
Collybia butyracea	B
Conocybe tenera	C
Coprinus comatus	C
C.micaceous	C
Coprinus sp.	-
Cortinarius sp.	-
Gymnopilus penetrans	-
Inocybe sp.	-
Laccaria laccata	-
Lacrymaria sp.	-
Lactarius rufus	A
Lepiota procera	C
Marasmius oreades	A
Omphalina sp.	-
Paneolus sp.	B
Paxillus involutus	-
Russula ochroleuca	C
Russula emetica	C
Tricholoma saponaceum	-
T.terreum	-
Tubaria furfuracea	C
b. Gasteromycetales	
Lycoperdon sp.	-

A = browning of cap surface below inoculation drop

B = browning and sinking of tissue below inoculation drop

C = surface development of Verticillium mycelium

D = browning and collapse of tissue associated with Verticillium mycelium

- = no visible symptoms

Symptoms were scored after 24 h incubation at room temperature.

Only 2 species, A.arvensis and A.silvicola, displayed symptoms similar to those produced on infection of A.bisporus. As judged by lesion development however, the pathogen was less aggressive on these hosts than on A.bisporus. On a third species, A.macrosporus, surface mycelium of V.fungicola developed in the absence of tissue collapse.

Intra-generic differences in the response to inoculation were also observed with Coprinus spp. Both C.micaceous and C.comatus supported surface development of V.fungicola whilst with the unidentified species there was no response. Although not examined in every instance, the absence of a reaction to inoculation with V.fungicola often coincided with a lack of conidial germination on the sporophore pileus.

The single member of the Gasteromycetales (Lycoperdon sp) examined gave no reaction when inoculated with V.fungicola.

No Verticillium species were isolated from the rust and powdery mildew fungi collected during the summer of 1980 (Table 39). However. inoculation of rust fungi with V.fungicola resulted within 5 days in an abundant sporulating aerial growth of the pathogen on the pustules of all six species tested. A similar prolific growth of V.fungicola also occurred after inoculation of 3 species of powdery mildew (Table 40).

A further examination of the interaction between V.fungicola and S.fuliginea showed that the aerial powdery mildew mycelium was extensively colonised and that the mildew conidia collapsed when contacted by V.fungicola hyphae (Plates 54a & 54b). In the absence of the powdery mildew the development of V.fungicola on the leaf surface was limited. Approximately 30% of the conidia had germinated after 48 hours but germ tubes were short and there was little further development after 5 days. Around broken leaf hairs, from which nutrients are likely to have exuded, development was more prolific which suggests that the lack of germination on the leaf lamina may have been due to nutrient deficiency.

a

b

Plate 54 a and b Hyphae of V.fungicola contacting and causing collapse (arrowed) of S.fuliginea conidia on a cucumber leaf surface. a x 200, b x 400

Table 39. Rust and powdery mildew fungi examined for the presence of *Verticillium* species.

Species	Host	Isolation
Rusts		
Puccinia lagenophora	*Senecio*	-
P.malvacearum	*Malva*	-
P.punctiformis	*Cirsium*	-
Tranzschelia pruni-spinosi	*Anemone*	-
Powdery mildews	ex *Brassica*	-
	ex *Digitalis*	-
	ex *Epilobium*	-
	ex *Fumaria*	-
	ex *Plantago*	-
	ex *Polygonum*	-
	ex *Ranunculus*	-
	ex *Rosa*	-
	ex *Sonchus*	-
	ex *Stachys*	-

- = no *Verticillium* species isolated.

There was no interaction between *V.fungicola* germ tubes and mildew conidia on glass slides. The germ tubes remained short and showed no tropic response towards the conidia. Contacted conidia also showed no evidence of the collapse which was frequently observed on the leaf surface.

The sterile leachate of mildew conidia stimulated the germination of washed *V.fungicola* conidia compared to the control, although the difference was not statistically significant ($P = 0.05$) (Table 41).

Table 40. The effect of inoculating rust and powdery mildew fungi with suspensions of V.fungicola.

	Spore type	Host	Reaction
Rusts			
Melampsora larici-populi	U	Populus	+
Puccinia lagenophora	U	Senecio	+
P.malvacearum	U	Malva	+
P.punctiformis	U	Cirsium	+
P.striiformis	U	Agrostis	+
Uromyces dianthae	U	Dianthus	+
Powdery mildews			
Erysiphe cichoracearum		Plantago	+
E.graminis		Hordeum	+
Sphaerotheca fuliginea		Cucumis	+

\+ = growth of V.fungicola visible within 5 days

U = urediniospores

Table 41. The effect of a leachate of conidia of the powdery mildew Sphaerotheca fuliginea on the germination of washed conidia of V.fungicola. Germination was assessed after 24 h incubation at 20° as the mean of 3 replicates per treatment.

	Mean % germination	SE
+ mildew conidia leachate	65.5	8.25
+ leaf surface washings (control)	39.3	8.89

Calculated t value = 2.15
Tabulated t value for 4 degrees of freedom ($P = 0.05$) = 2.77

When whole cucumber plants infected with mildew were inoculated with a V.fungicola conidial suspension, the development of V.fungicola was limited and there was no obvious evidence of control of the mildew. Three days after inoculation, V.fungicola had developed in isolated patches and after a further 3 days had only spread to give foci of 0.5 - 1.0 cm diameter. Within the foci the leaf surface was largely clear of mildew which appeared to have been lysed by V.fungicola.

No colonies of V.fungicola developed on the plates of selective medium over which infected mildew colonies and discs of V.fungicola agar culture had been tapped.

Discussion

Verticillium species have been isolated from several species of wild Basidiomycetes (Gams, 1971; Preuss, 1851; Wakefield & Bisby, 1941) but the limited survey of diseased sporophores performed here suggests that Verticillium species are not of widespread distribution in this ecological niche. The results confirm those of Cross (1971) who, in a similar attempt to establish the host range of V.fungicola, also failed to isolate the pathogen from a number of specimens of observed Basidiomycetes.

The inoculation experiments suggest that even under favourable environmental conditions, only a limited number of genera and species are susceptible to attack by V.fungicola. As might be expected, wild Agaricus species gave the most similar response to infection to A.bisporus, although A.macrosporus appeared to be relatively resistant. It should be borne in mind however that the reaction of cut sporophores to inoculation with V.fungicola may not necessarily correspond to the reaction of living sporophores.

When several of the species examined were inoculated with V.fungicola the pathogen developed only as surface mycelium and there was no tissue

collapse. This is analogous to the development of V.lamellicola on cut A.bisporus sporophores on which the fungus appears to be able to colonise the surface tissue without formation of lesions but is unable to incite disease on growing sporophores (Gandy, 1973). Species capable of surface growth alone on cut sporophores may be viewed as saprophytes colonising a nutritionally favourable substrate rather than as true pathogens.

The browning and tissue collapse in the absence of mycelial development that was noted only with the species of Paneolus may have been due to bacterial rather than fungal attack.

These observations throw little light on the possible factors that determine the host range of V.fungicola within the Agaricales but it is interesting to note that with several species which gave no reaction when inoculated, conidia failed to germinate on the sporophore surface. This suggests that in some instances resistance may operate through a chemical barrier to infection, perhaps due to a limited nutrient supply or the production of antibiotics.

There have been numerous references to associations between Verticillium spp. and rust fungi (see Table 37) although the failure to isolate them from the range of rusts examined again suggests that they are not of very widespread distribution in this habitat.

All of the rust species tested were overgrown by V.fungicola although parasitism was not clearly demonstrated. The colonisation of rust pustules by V.fungicola is analogous to that described for V.lecanii which attacks and also controls Uromyces dianthae (Pers) Niessl on carnation (Spencer, 1980). Although V.lecanii has been shown to cause the lysis of germ tubes of 11 species of rusts (Garcia-Acha et al, 1965), the urediniospores of Uromyces dianthae appear to be directly penetrated by the pathogen (Spencer & Atkey, 1981). It seems likely that both forms of parasitism may occur but perhaps at different developmental

stages of the host fungus.

There are fewer references to Verticillium species attacking powdery mildew fungi although the effects of parasitism are perhaps more clearly seen than with rusts.

When cucumber powdery mildew was inoculated with a V.fungicola conidial suspension, areas of the mildew colonies became degenerate because of lysis of both conidia and mycelium. An analogous association has been described by Hall et al (in preparation) for V.lecanii and Erisyphe graminis. These authors also reported that V.lecanii commonly colonised powdery mildews of several grass species in the wild. These observations underline the physiological as well as morphological similarities between V.fungicola and V.lecanii.

The relationship between V.fungicola and cucumber powdery mildew appears to be nutritional since conidia germinate and develop poorly on the leaf surface in the absence of the mildew. Also, the exudate of mildew conidia promoted the germination of V.fungicola conidia although the characteristic collapse of the mildew conidia observed on the leaf surface could not be reproduced in vitro. The close association between V.fungicola and the mildew on the leaf surface suggests that the transfer of nutrients is direct although it is possible that the mildew promotes leakage of nutrients from the leaf which may also influence the development of V.fungicola.

The spread of V.fungicola over cucumber powdery mildew colonies on whole plants was limited under laboratory conditions and there was no apparent control of the disease since the mildew eventually colonised the whole plant. This is surprising in view of the destructive nature of the interaction on leaf discs and the large amount of host material available for colonisation. It may be that the failure to control the mildew was due to resistance or unsuitability of the host or perhaps to poor dispersal

of V.fungicola within the chambers in which water splash was kept to a minimum. Biological control of the rust U.dianthae on glasshouse carnations by V.lecanii has recently been reported by Spencer & Atkey (1981).

e. Insects

The close morphological similarity between V.fungicola and the insect pathogen V.lecanii and the overlap in the ecological niches they occupy (see Table 27) suggests that there may also be some overlap in their host ranges.

The pathogenicity of V.lecanii C3 isolated from the aphid Macrosiphoniella sanbornii (Gilette) towards cut sporophores has been previously demonstrated to depend on inoculum concentration but there was no evidence of tissue deformation when growing sporophores were inoculated with this isolate (see Pathogenicity Test section).

Attempts were made to demonstrate the pathogenicity of V.fungicola isolate G3 towards two species of aphid and to isolate Verticillium spp. from insects trapped in the wild.

Materials and Methods

Insects were trapped during two separate periods; during October and November 1980 and February and March 1981. In the earlier sampling period the insects (which were mainly small flies resembling sciarids) occurred as contaminants of the spore trap slides (see following section for description of spore traps). During the latter period, insects were trapped on two 15 x 15 cm squares of yellow plastic which were coated with a polybutene adhesive and fixed to the sides of a tray of spawn-run mushroom compost. The tray was situated 100 m from the Institute mushroom unit, adjacent to the belt of deciduous woodland. The traps were changed weekly.

Insects from both sets of traps were removed and placed on plates of selective medium which were incubated at 20°.

The pathogenicity of V.fungicola G3 towards two aphid species Macrosiphoniella sanbornii and Rhopalosiphum padi (Hall,R. pers. comm.) was tested using the method of Hall (1976). Both species are susceptible to V.lecanii. Adult aphids were briefly immersed in a suspension of V.fungicola isolate G3 conidia in 0.01% Triton X 100 (c. 1 x 10^7 ml^{-1}) prepared from 7 day old PDA cultures. Control aphids were immersed in Triton X 100 alone. After treatment, single aphids were placed on 1 cm diameter leaf discs of either Chrysanthemum (M.sanbornii) or wheat (R.padi) which were laid on water agar in the compartments of multi-chambered Perspex trays. The trays were sealed with a sheet of polythene and a small hole was pierced above each chamber. The trays were incubated at 24° with an approximately 12 h photoperiod under white fluorescent lights. The number of aphids used for each treatment was 25 (1 tray) in the M.sanbornii assay and 75 (3 trays) in the R.padi assay. After 5 days, the numbers of alive and dead aphids and those associated with Verticillium were counted.

Results

Five isolates of Verticillium were obtained from insects trapped during October and November 1980. These varied in colony type from felted (similar to isolates of V.lecanii identified by W.Gams) to cottony, but none of the isolates were satidfactorily identified. All of the isolates were included in the in vitro pathogenicity assay but none achieved a score of 8 or above. None of the isolates were included in the field pathogenicity assay.

During the later sampling period, few insects were trapped because of the low temperatures and none yielded isolates of Verticillium.

The results of the aphid pathogenicity assays are given in Table 42. Under the conditions of these assays, pathogenic isolates of V.lecanii (eg. C3) generally give over 90% mortality on a susceptible host species and the mortality in controls is usually less than 5% (Hall, 1976a).

In both the aphid pathogenicity assays there was an unsatisfactorily high mortality in the uninoculated controls (approximately 80%). In spite of contamination, however, over 3 times as many dead aphids were associated with Verticillium in the inoculated treatment compared to the controls in the M.sanbornii assay. In the R.padi assay none of the controls were contaminated but only a small proportion of the dead aphids in the inoculated treatment were associated with Verticillium.

Discussion

Insect pests of cultivated mushrooms are able to spread V.fungicola both under laboratory conditions and within the crop (Cross & Jacobs, 1969; White, 1981) but the possible role of wild flies in initiating primary outbreaks of the disease has not been previously investigated.

The limited survey of Verticillium species associated with insects in the wild did not result in the isolation of a mushroom pathogenic strain and all isolates obtained must therefore be excluded from V.fungicola. This suggests that even if the pathogen is able to exploit insects as a habitat, the phenomenon is not of very widespread occurrence.

The high mortality in the M.sanbornii assay controls was partially accounted for by the contamination of the controls with a Verticillium species of uncertain identity (although distinct from V.fungicola G3) which may have occurred during inoculation or may have been due to an endemic infection in the aphid stock. The reason for high control mortality in the R.padi assay is not clear but may possibly be due to factors such as drowning during treatment or to suboptimal environmental conditions.

Table 42. The pathogenicity of *V.fungicola* isolate G3 towards the aphids *Macrosiphoniella sanbornii* and *Rhopalosiphum padi*. All counts were made 5 d after treatment. The figures for *R.padi* are the means of 3 replicate 25 aphid samples in each treatment.

	M.sanbornii		*R.padi*	
	Inoculated	Control	Inoculated	Control
Initial No. of aphids	25	25	25	25
Total No. of aphids after 5 d	53	54	53	46
Total No. alive	11	9	10	11
Total No. dead	42	45	44	35
Total No. alive visibly infected with *Verticillium*	3	2	0	0
Total No. dead visibly infected with *Verticillium*	18	6	2	0
% of total No. of aphids dead	79	83	82	77
% of total No. of aphids associated with *Verticillium*	40	15	3	0
% of total No. of dead aphids associated with *Verticillium*	43	13	4	0

Because of the high mortality in the controls, the low replication and small sample numbers used, the conclusions that may be drawn from the aphid pathogenicity assays are necessarily limited. The results suggest however that *V.fungicola* isolate G3 may be mildly pathogenic towards *M.sanbornii* but not towards *R.padi*. Thus, as well as attacking a range

of fungi, V.fungicola may also be induced to attack aphids under laboratory conditions. However, the pathogenicity of the isolate tested was much less than that of pathogenic isolates of V.lecanii. Within this species however there are marked variations in pathogenicity and not all isolates are pathogenic towards insects. For example, Hall (in preparation) demonstrated that of 8 isolates of V.lecanii obtained from rust and powdery mildew fungi which were tested for pathogenicity towards M.sanbornii, 2 were non-pathogenic, 3 showed intermediate and atypical pathogenicity and 3 were as pathogenic as isolates obtained from diseased aphids. However, Hall et al (in preparation) failed to consistently infect aphids with 5 other V.lecanii isolates also obtained from plant pathogenic fungi and therefore concluded that there is a degree of physiological specialisation as regards host range within the species.

These authors also observed large differences in the infectivity of a single isolate between replicate experiments which emphasises the variability encountered in these bioassays.

Since isolates included within V.lecanii show some ability to attack other fungi as well as a variable ability to attack insects it may be that there is a similar degree of variability in host range within V.fungicola. The similarities between V.fungicola and V.lecanii clearly extend beyond morphology to physiology, ecology and perhaps pathology and the limits of the two species are therefore not precisely defined. This renders the distinction of the two species of little practical taxonomic value and further comparative studies of the ability of isolates of V.fungicola from mushrooms and V.lecanii from insects and fungi to attack a range of host organisms may help to elucidate the confused taxonomy of these species.

f. Spore trapping

Attempts were made to trap airborne V.fungicola propagules during two separate periods; from March to October 1979 using a Hirst automatic volumetric spore trap (HAVST, Hirst, 1952) and from October to November 1980 using exposed sticky slides.

The HAVST was placed 0.65 m above ground level near the GCRI mushroom isolation unit (see Fig. 20). Initially, spores were impacted onto microscope slides coated with petroleum jelly, but small conidia were difficult to distinguish and, [illegible] because of a lack of distinctive features, were impossible to identify. Consequently, slides were coated with a polybutene adhesive (Hyvis 200) which is normally used for trapping insects and which was shown to be non-toxic to V.fungicola conidia in germination tests. After exposure in the spore trap for 24 hours (flow rate, 10 l min^{-1}) the slides were rotated, sticky face down, on two plates of selective medium and were then placed sticky side down on a third plate of medium. The slides were removed and discarded after 5 days and the plates incubated and examined as described previously.

This method was tested by attempting to recover V.fungicola from artificially infested dust. Air dried soil (GCRI cultivated field site) was rewetted to 50% water holding capacity with a washed V.fungicola conidial suspension giving a final concentration of c. 2×10^4 conidia g^{-1} air dry weight. The soil was re-dried at room temperature, ground and passed through a 500 µm sieve. About 0.5 g of the infested dust was sprinkled 10 cm in front of the spore trap inlet on 4 consecutive days and the slides were treated as described above.

During the second period of trapping, microscope slides coated with polybutene adhesive were exposed for 24 hours in the modified HAVST slide holder from which the outer casing and inflow nozzle had

been removed and in the housing of a mushroom cropping house ventilating fan, 4 m above the ground facing the predominant wind direction (see Fig.20). In both cases the slides were protected from the rain, although insects and particles of dust were not excluded. After exposure the slides were treated as described for those from the operating HAVST. An attempt was made to correlate the incidence of Verticillium species in the air with soil and air temperature, rainfall and relative humidity from records taken at the GCRI.

Results

Despite the large local release of inoculum when Verticillium infested dust was sprinkled in front of the spore trap inlet, few Verticillium colonies developed on the plates of selective medium. This suggests that the efficiency of recovery of inoculum using this technique was low and whilst the method was therefore of limited use in the quantitative estimation of airborne Verticillium inoculum, its main advantage lay in the provision of living cultures from which identifications could be attempted.

During the March-October 1979 period of spore trapping using the HAVST, six isolates of Verticillium were obtained, four of which were isolated in August. One isolate (ST 29) was identified by W. Gams as V.lecanii whilst two others (ST 19 and 20) were morphologically similar to V.psalliotae.

During the October-November 1980 period of trapping, using exposed sticky slides, 5 isolates of Verticillium were obtained from the fan housing trap and 5 from the modified spore trap. Half of these isolates were associated with insects that had become trapped on the unprotected slides. None of these isolates were morphologically typical of either the insect pathogen V.lecanii or of V.fungicola.

All 16 isolates were included in the in vitro pathogenicity test

but none proved to be pathogenic. Isolates ST 24 and ST 29 from the HAVST were also included in the field pathogenicity test. Neither isolate produced deformities of mushroom tissue characteristic of V.fungicola although ST 29 may have caused a certain amount of cap spotting. This has since been confirmed by W C Wong (pers. comm.)

Discussion

The spore trap results indicate that inoculum of Verticillium species may be disseminated by air currents although none of the isolates recovered were pathogenic to mushrooms and therefore the possibility of air dispersal of V.fungicola inoculum remains uncertain.

Too few isolates were obtained in this study to be able to draw conclusions about the factors that affect aerial dispersal of Verticillium inoculum. Most of the isolates were recovered several days after a period with limited or no rainfall but these conditions are generally conducive to the liberation and dispersal of fungal propagules.

Because of the lack of distinctive characters of Verticillium conidia, there have been few reports of their occurrence in the air spora. However, Darke et al (1976) reported that from 1 - 10% of spores trapped close to an operating combine harvester were of a 'Verticillium/Paeciliomyces' type and that V.lecanii was very common. This is interesting in view of the ability of this species to attack cereal rusts and aphids, both of which can be abundant in cereal fields and suggests that the leaves of cereals may be a common habitat of this fungus.

If aerially dispersed inoculum of Verticillium is in the form of conidia, it is likely to be protected from desiccation by association with particles of debris since the conidia of all Verticillium species are probably as susceptible to low relative humidity as are those of V.fungicola. Other possible forms of inoculum include mycelial fragments and resting structures such as chlamydospores.

CHAPTER 7

BENOMYL INSENSITIVITY IN V.FUNGICOLA AND VERTICILLIUM ISOLATES FROM NATURAL SUBSTRATES.

Insensitivity of V.fungicola to benomyl rapidly became widespread after the introduction of the fungicide for dry bubble control (Bollen & van Zaayen, 1975; Fletcher & Yarham, 1976). Wuest et al (1974) suggested that benomyl insensitive strains occur in the natural environment from which large insensitive populations develop under the selection pressure of widespread fungicide use. The isolation of Verticillium species from natural substrates provided an opportunity to study the distribution of benomyl insensitivity in wild populations of Verticillium species.

Materials and Methods

1. Experiments with benomyl amended media.

The basal medium used in these studies was PDA, prepared and autoclaved in 100 ml amounts. Because of the poor solubility of benomyl in water, amendments to the medium were made with the active product derived from benomyl, carbendazim, dissolved in hydrochloric acid. Concentrations were expressed as benomyl equivalents (BE).

A stock solution of carbendazim was prepared by dissolving 6.7 g of Bavistin in 100 ml of 0.01N HCl; 1 ml of this solution was added to 100 ml of molten PDA to give 500 $\mu g\ ml^{-1}$ BE. Control media were prepared by adding 1 ml of 0.01N HCl to 100 ml molten agar.

To determine the ability of 38 Verticillium isolates to grow on 500 $\mu g\ ml^{-1}$ BE amended PDA, plates were inoculated as described in the General Methods and the results were recorded as either + or - growth after 7 d incubation at 20°C.

The proportion of insensitive strains occurring naturally in wild populations was studied by spreading conidial suspensions over the surface of 500 μg ml^{-1}BE amended PDA (c. 10^7 conidia $plate^{-1}$). The benomyl sensitive strains used, namely ST29 from a spore trap catch and P94 from peat, were morphologically very similar to V.fungicola but were subsequently identified as V.lecanii by Dr.W Gams. After 7 d incubation at 20°C, insensitive colonies were counted and the frequency of insensitive strains calculated assuming that each colony arose from a single conidium. A sample of the insensitive colonies was transferred to unamended PDA and some were also returned to 500 μg ml^{-1}BE amended PDA to check their stability on this medium. The linear growth on unamended PDA, morphology and pathogenicity of the sensitive isolates and the corresponding insensitive strains were compared.

2. The production of acid in liquid culture.

Lambert and Wuest (1977) suggested that the basis for benomyl insensitivity in V.fungicola might be the production of acid by insensitive strains. This possibility was investigated by comparing acid production by 5 sensitive (V.fungicola G3, V.lecanii C3, ST29s, P94s and V.psalliotae C9) and 3 insensitive (V.lecanii ST29/5, ST29/6 and P94/10) isolates. Two replicate 250 ml flasks, containing 100 ml of sterile Lambert and Wuest medium A, were each inoculated with two 6 mm diameter discs cut from a 7 d old culture of the appropriate isolate. The pH of the culture filtrates was measured after 7 d static growth at 20°C and compared with that of the uninoculated medium.

Results

1. Experiments with benomyl amended media.

The responses of the 38 Verticillium isolates are shown in Table 43.

Table 43. Growth of *Verticillium* isolates on PDA amended with 500 $\mu g/ml^{-1}$ BE after 7 days at 20°.

Isolate	Identity	Source	Growth on benomyl amended agar
S1		Mushrooms	+
G3		"	+
G4	*V.fungicola*	"	+
G5		"	+
C1		"	+
P1		"	+
C9		"	-
P219		Peat	+
P371/2		"	+
P337		"	+
P402/7	*V.psalliotae*	"	+
P398		"	+
P335		"	+
P372		"	+
ST19		Spore trap	+
R110		Soil	+
R18		"	+
K3		"	+
P367	*V.bulbillosum*	Peat	+
P353		"	-
P267		"	+
P276		"	+
P186		"	+
Aphid 1		Aphid	-
Aphid 2		"	-
C3		"	-
P94		Peat	-
P72		"	-
P193	*V.lecanii*	"	-
P176		"	-
P122		"	-
P215		"	-
P271		"	-
ST29		Spore trap	-
LL33		Leaf litter	-
LL72	*V.lamellicola*	"	-
P282		Peat	+
CH1	*V.leptobactrum*	Chalk	-

\+ Growth
\- No growth

All 12 V.lecanii strains were sensitive, but 8 of 9 V.psalliotae, 7 of 8 V.bulbillosum and all 6 V.fungicola isolates were insensitive.

The recovery rates of insensitive strains from the two sensitive V.lecanii isolates were 1 in 6 x 10^6 for ST29 and 1 in 9 x 10^6 for P94. The effect of benomyl on conidia was fungistatic, growth being halted after germination but proceeding normally on transfer to unamended PDA. Linear growth rates of the insensitive strains were reduced compared to those of the comparable sensitive isolates (Fig 26), There were few differences in morphology between the insensitive and sensitive strains, phialide length being the most variable character, Neither the original isolates nor the derived strains were pathogenic to cut or growing sporophores.

2. Acid production as a basis for insensitivity.

The mean pH of the cultures after 7 days is shown in relation to the control in Figure 27. The pH of the medium increased with the sensitive isolates G3 and ST29s but decreased with all the others irrespective of sensitivity.

DISCUSSION

Insensitivity to benomyl is widespread in wild populations of some Verticillium species (eg. V.psalliotae, V.bulbillosum) although absent in others (eg. V.lecanii). The isolates of V.fungicola examined were obtained from mushrooms after the introduction of benomyl for dry bubble control and were all insensitive. However, Wuest et al (1974) described an insensitive, although morphologically atypical, isolate (ML2) which was obtained prior to the discovery of carbendazim fungicides which suggests that insensitivity occurs in wild populations

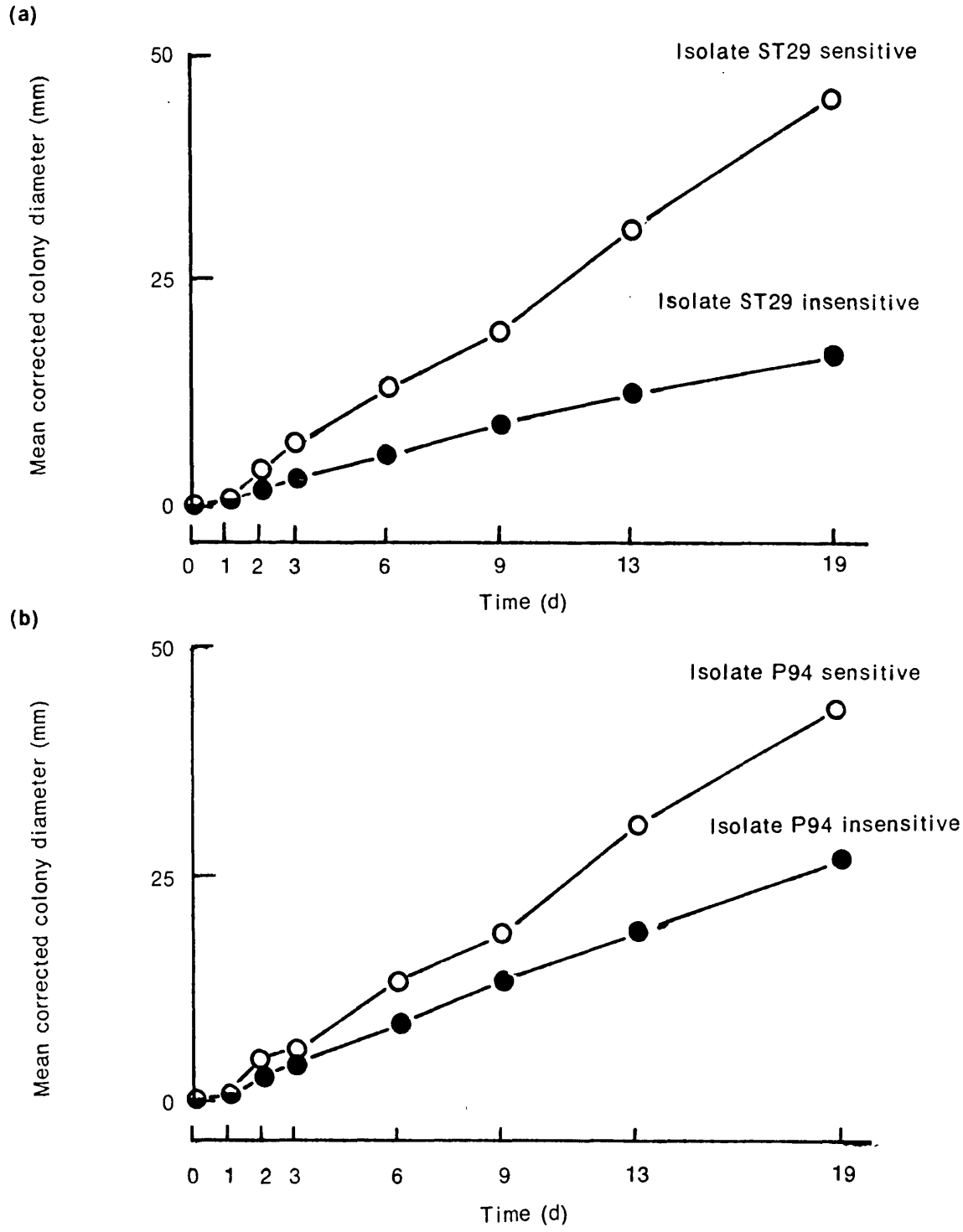
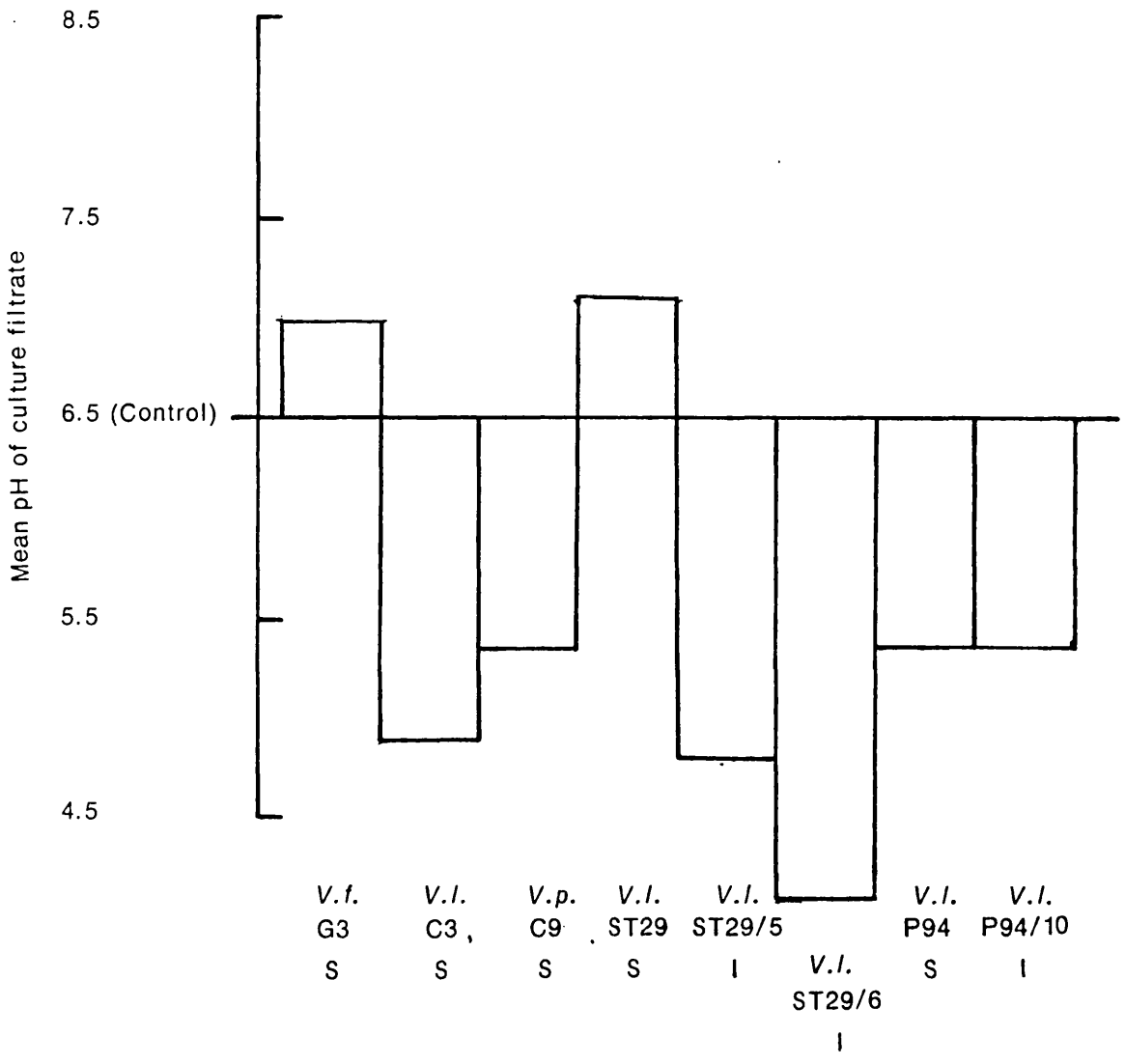


Fig.26. Mean corrected linear growth (mm) of benomyl sensitive and insensitive strains of isolates (a) ST29 and (b) P94 on PDA at 20°C.



S : sensitive

I : Insensitive

V.f. *V. fungicola*

V.l. *V. lecanii*

V.p. *V. psalliotae*

Fig.27. pH changes in culture filtrates of *Verticillium* isolates relative to uninoculated controls in relation to benomyl sensitivity. pH measurements were made after 21d incubation at 20°C.

of the pathogen. The reason for the existence of genes that code for insensitivity to benomyl and the polymorphism within species in wild populations is unclear, although the phenomenon appears to be fairly common. Insensitivity has been reported in populations of Ceratocystis ulmi (Buisman) C. Moreau (Schrieber & Townsend, 1976), Sphaerotheca fuliginea (Dinoor cited in Wolfe, 1975) and Cercospora beticola Sacc. (Dovas et al, 1975) occurring in regions geographically isolated from sites of benomyl use.

In the presence of benomyl in vitro, the recovery of insensitive strains from populations of the two sensitive V.lecanii isolates ST29s and P94s were high, especially when the vast number of conidia produced was taken into consideration. Under similar selection conditions, Brasier and Gibbs (1975) found that the recovery rate of insensitive strains in C.ulmi was 1 in 10^8 conidia. Insensitivity to benomyl can be readily demonstrated in populations of several fungi in vitro either by exposure to the fungicide alone or by mutagenic treatment followed by selection. Van Tuyl (1975) for example, treated sensitive strains to UV irradiation and induced insensitivity in all 10 fungi tested.

The number of insensitive strains recovered from benomyl amended agar may either represent the proportion of insensitive strains in the wild population or the rate of mutation to insensitivity in the presence of benomyl. These two sources of insensitivity cannot be separated because insensitivity can only be identified in the presence of the fungicide which is itself a mutagen (Hastie, 1970).

Selection pressure alone does not invariably result in the formation of insensitive strains either in vitro (Ruppel, 1975 with Cercospora beticola) or in the field after persistent exposure to the fungicide (Fletcher & Yarham, 1976, with Mycogone perniciosa; Tate et al, 1974, with Monilinia spp.) and the factors governing the formation of insensitive strains are unknown.

In V.fungicola and other fungi the growth rates of benomyl insensitive isolates are often reduced compared to those of sensitive isolates (Fletcher & Yarham, 1976; Richmond & Pring, 1980). The two sensitive isolates of V.lecanii tested here grew more slowly than their corresponding insensitive strains. Further evidence of reduced fitness in insensitive isolates of V.fungicola in vitro was reported by Wuest et al (1974) for isolate ML2 in which growth rate, spore production and spore viability were less than in sensitive isolates. This isolate was also less pathogenic towards cut mushrooms than sensitive isolates both in the presence and absence of benomyl. However, in the field, morphologically typical insensitive isolates are usually as pathogenic as, or occasionally more pathogenic than, sensitive isolates in terms of crop loss despite slower growth rates in vitro (Bollen & van Zaayen, 1975; Fletcher & Yarham, 1976). Neither ST29, P94 nor any of the corresponding insensitive mutants were obviously pathogenic towards either cut or growing mushrooms.

No work has been done on the relative fitness of sensitive and insensitive strains of V.fungicola to survive in the absence of the mushroom crop or benomyl, factors which are likely to determine whether or not benomyl will possibly be of use again in the future. Subsequent to the comparable rapid spread of benomyl insensitivity in Cercospora beticola in Northern Greece and the USA, (Dovas et al, 1976; Ruppel et al, 1980) there has been no evidence of a decline in the benomyl insensitive population, which appears equally able to survive as the sensitive population under field selection conditions. It seems possible that with V.fungicola also, the large and continuous selection pressure on insensitive individuals during the period of widespread benomyl use may have been sufficiently intense to cause a shift in the whole population towards a balanced insensitivity, in which case a decline in insensitive strains is unlikely (Wolfe, 1975)

Lambert and Wuest (1977) found that 4 benomyl insensitive isolates of V.fungicola grown in liquid culture reduced the pH of the culture filtrate by an average of 2.1 units after 7 days compared with uninoculated controls, whilst sensitive isolates tended to increase the pH. A reduction in the pH of culture filtrates of a similar magnitude was recorded with the V.lecanii insensitive strains ST29/5 and ST29/6, whilst the corresponding sensitive isolate increased the pH of the culture filtrate, thus supporting Lambert and Wuest's suggestion that the production of acid is correlated with benomyl insensitivity. However, whilst all the insensitive isolates produced acid, several of the sensitive isolates did also and, with V.lecanii P94, the sensitive strain and the corresponding insensitive isolate both reduced the pH to the same extent. In V.lecanii and V.psalliotae therefore, acid production and benomyl insensitivity are not invariably linked. Similar studies using sensitive isolates of V.fungicola and corresponding insensitive isolates would provide further evidence for the validity of the hypothesis.

CHAPTER 8

GENERAL DISCUSSION

One of the aims of this research was to elucidate the mechanisms of pathogenesis of V.fungicola. The symptoms resulting from infection of mushroom sporophores have long been recognised to be dependent on the stage of development at infection; infection of fully expanded sporophores gives rise to necrotic surface lesions whilst infection prior to this results in deformities ranging from localised swelling to masses of undifferentiated tissue.

In this study, both the microscopic observations and the demonstration of the ability of the pathogen to degrade mushroom cell walls and their constituent polymers in vitro substantiate the suggestion (Ware, 1933; Vincent-Davies, 1973) that the symptoms of infection of mature sporophores are due to the production of extracellular enzymes by the pathogen. The tissue extract experimants indicated that close contact between the host and pathogen was required for the production and isolation of the degradative principle as it was not possible to demonstrate extracellular enzyme production by the pathogen alone. Since A.bisporus has been shown to possess enzymes capable of degrading its own cell walls (Vincent-Davies, 1973), the possibility exists that tissue collapse may in part be due to a stimulation of the wall degrading enzymes of the host itself. However, extracellular cell wall degrading enzyme activity has been demonstrated in the similar mushroom pathogen Mycogone perniciosa (Vincent-Davies, 1973) and the lack of activity in V.fungicola culture filtrate may have been due to unsuitable cultural conditions.

Several non-pathogenic species of Verticillium also showed some

ability to degrade the mushroom cell wall preparation. If these results reflect the degradative ability of the isolates when in contact with living mushroom tissue, it suggests that the ability to degrade mushroom cell walls does not primarily determine host specificity. The factors which do control host specificity in Verticillium-Agaricus interactions however remain unclear.

Although the symptoms produced on infection of expanding sporophores are different from those produced on mature sporophores, the mechanisms governing their production may be similar. The rapid growth of sporophores is primarily due to expansion of previously differentiated cells (Craig et al, 1977) and to accommodate this expansion, the cell walls must increase in plasticity. This necessitates a loosening of the cell wall structure which is thought to be brought about by a loss of β glucan and an opening of the chitin network (Michalenko et al, 1976; Vincent-Davies, 1973). It would seem likely, therefore, that the interaction of pathogen derived wall degrading enzymes with an already loosened wall structure could cause further wall plasticity and consequent loss of control of cell expansion.

V.fungicola was shown to stimulate the elongation of rapidly expanding stipe cells and, since interactions between cells are likely to be of importance in determining tissue differentiation and the final form of the fruit body, this mechanism may account for the swellings and deformities characteristic of infection of sporophores during their phase of rapid expansion.

Some of the differences in symptoms produced by infection at different developmental stages may, therefore, reflect the different physiological and structural states of the mushroom cell wall at infection. Furthermore, it is also possible that the inability of V.fungicola to attack vegetative mushroom mycelium is due to differences

in the composition and conformation of wall polymers in vegetative and sporophore hyphae (Vincent-Davies, 1973).

Other symptoms (eg. stunting) may however be more readily explained by the failure of parts of the sporophore to develop normally. This could also be brought about by disruption of cell wall structure perhaps either at a critical point in the development process, causing an interruption of the flow of metabolites to the expanding region, or by interfering with the production and utilisation of growth regulators (Gruen, 1963).

The second aim of this study was to investigate the ecology of V.fungicola in an attempt to establish likely sources of inoculum as an aid to disease control.

Verticillium species have been isolated in micro-ecological studies of diverse habitats using a range of synthetic media but whilst the selective medium developed for this purpose did not give entirely satisfactory quantitative results, it was successfully used in isolating Verticillium species from all the habitats examined. This medium could therefore advantageously be used in further investigations of the ecology of Verticillium.

This study indicates that Verticillium species are of widespread occurrence in natural substrates. Although many of the isolates obtained were morphologically similar to V.fungicola, none of those tested could be unequivocally included within the species on the basis of pathogenicity. However, the in vitro pathogenicity test, whilst convenient for screening a large number of isolates, was essentially arbitrary and may not have reflected true pathogenic capabilities of an isolate. The critical test of pathogenicity is the production of the whole range of symptoms characteristic of dry bubble on mushroom beds but, because of space limitations, relatively few isolates were tested in this way. Apart from the two

V.fungicola controls, none of the isolates included in the field pathogenicity test reproduced the entire disease syndrome. Several of the isolates morphologically most similar to V.fungicola, (but included within V.lecanii by W Gams) caused some spotting of pilei, however. The three isolates which gave high scores in the in vitro pathogenicity test were not included in the field test and their pathogenicity therefore remains uncertain.

The review of the literature suggested several possible sources of the pathogen: there are records of isolates morphologically corresponding to V.fungicola from soil, leaf litter and fungi. In none of these reports was pathogenicity towards mushrooms demonstrated and the inclusion of isolates within V.fungicola on morphology alone now seems questionable. The reports of V.fungicola-like isolates from wild Basidiomycete fruit bodies is especially intruiging because of the similarity between hosts and between this habitat and the mushroom crop. Both pathological and ecological aspects of interactions between Verticillium species and other fungi merit further investigation.

Perhaps the most interesting observation in this study of the ecology of Verticillium species was the isolation of the pathogenic species V.psalliotae from horticultural peat. In view of the application of this substrate to mushroom beds as a constituent of casing, further studies of the distribution of V.psalliotae in peat may prove epidemiologically important, particularly if the commercial cultivation of A.bitorquis (the favoured host of this pathogen) becomes widespread.

As discussed in Chapter 6, research into the ecology of Verticillium species is greatly hindered by the lack of knowledge of the taxonomy of the genus, especially as regards species limits and the morphological variation within species. Observations in this study suggest that both morphological and physiological intermediates exist between several

currently accepted species which renders the taxonomic schema of Gams (1971) of little practical value. At present there is no alternative taxonomic treatment of the genus and much work needs to be done in this field. Promising supplementary approaches to conventional taxonomy that may further our understanding of this morphologically variable group of fungi include gel electrophoresis or isoelectric focussing of mycelial extracts (Milton et al, 1971; Pelletier & Hall, 1971), serology (Fitzell et al, 1980) and numerical taxonomy.

The question of the identity of possible sources of inoculum of dry bubble disease therefore remains uncertain and is likely to do so until either the genus Verticillium receives a thorough, practically applicable taxonomic treatment or a suitable pathogenicity assay for screening large numbers of isolates is devised and employed.

From the limited results of this research it would be unwise to discount any of the substrates investigated as potential sources of inoculum although it suggests that they are unlikely to be common sources. Natural substrates may however still be an important source of inoculum for primary outbreaks of dry bubble. With most plant diseases the sources of particular epidemics are unknown but it is generally assumed that inoculum originates from other infected crops in the locality or is held over from one crop to the next. Only a small amount of inoculum is necessary to start an epidemic since the spread of many diseases in a susceptible crop is usually rapid under suitable environmental conditions. As with other diseases, primary outbreaks of dry bubble may be attributable to small amounts of inoculum from natural sources but after an initial outbreak has occurred, the majority of subsequent outbreaks are most likely to originate within the mushroom house or farm. An epidemic of

dry bubble produces a large amount of infective material and the continuous production of the mushroom crop and the practical difficulties in applying a stringent hygiene regime readily lend themselves to carryover of inoculum from diseased to healthy crops.

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POSTSCRIPT

Since the completion of the text of this thesis Gams and Van Zaayen (1982) have published further information on the taxonomy of fungicolous Verticillium species. They divide the genus Verticillium into 5 sections, two new sections (Nigrescentia, which includes the plant pathogenic species, and Albo-erecta, which contains mainly fungicolous species) supplementing the sections Erecta and Prostrata described by Gams (1972). A residual group of species remains to be described.

V.fungicola is redefined, the species being divided into three varieties. V.fungicola var.fungicola is pathogenic to A.bisporus causing dry bubble disease; V.fungicola var.aleophilum causes a spotting disease on both A.bisporus and A.bitorquis and is distinguished from var.fungicola by a higher maximum temperature for mycelial growth; V.fungicola var.flavidum is not pathogenic to A.bisporus or A.bitorquis and is defined by its lower optimum temperature for mycelial growth, pungent odour and occasional formation of sclerotia. This variety is frequently isolated from wild agarics.

Gams and Van Zaayen suggest that the use of temperature relationships as a taxonomic criterion within the complex V.fungicola leads to a sharper definition of the varieties than the variable morphological criteria allow. They further suggest that pathogenicity can be predicted by the response of isolates to a range of temperature.

Some observations on the response of pathogenic isolates of V.fungicola (V.fungicola.fungicola in Gams and Van Zaayen's scheme) to temperature were made in Chapter 3 of this thesis. Three isolates of V.fungicola were grown at temperatures ranging from 20 - 36°C on PDA and colony growth was measured after 18 days incubation. None of the isolates grew at 30° and all 3 showed a marked decline in growth between

24° and 27° (Fig 5) which supports the observations of Gams and Van Zaayen (their Fig 1). In contradiction to their results however all three isolates made some growth at 27°.

Lambert (1973) demonstrated that whilst pathogenic isolates of V.fungicola from regions with temperate climates showed little growth at 30°, isolates from regions with high mean summer temperatures grew well at this temperature. The use of temperature relations as a definitive taxonomic criterion must therefore be viewed with caution.

A second paper by Gams and Van Zaayen which examines the pathogenicity of the V.fungicola varieties is in press.

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